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Director
Prof. C. Pignata

PhD Thesis

*“Pathogenetic and diagnostic aspects in pediatric
hepatology and emerging complications of liver
transplantation”*

Tutor
Prof. P. Vajro

Student
Dr. M. Caropreso

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INTRODUCTION

The last decade has seen an explosion of activity in the clinical and research aspects of pediatric hepatology. The increased use of ultrasounds techniques and routine laboratory evaluation has drawn attention to several previously undiagnosed condition in childhood. The discipline has grown from a cataloguing of the many unique disorders that can occur during infancy and childhood to a more profound understanding of the genetic, biochemical, and virologic basis for many pediatric liver diseases.

Nowadays hepatopathies still represent a significant cause of morbidity and mortality worldwide. However advances in diagnosis and treatment, particularly the successful development of transplantation, have dramatically improved the outcome for infants and children with liver disease, so that many of them can now expect to grow into adult life. Sophisticated molecular genetic techniques have not only identified new genes and categorized rare defects, but have also given us an insight into pathophysiology and potential therapy. National and international collaboration through clinical database has helped us refine diagnosis and treatment of several pediatric hepatic diseases. Differently from adults, the signs and symptoms of liver disease are often non specific and can vary greatly from child to child among the different liver diseases. This is one reason for which it can be hard to diagnose. As a result, liver disease may frequently be overlooked. Because most childhood hepatic diseases are progressive and life threatening, diagnosis failure can have devastating consequences. At present for most childhood liver diseases the cause is still unknown and is poorly satisfactory. The recognition of the pathogenesis of liver disease, the implications of innovative diagnostic techniques and therapies, the investigations of new clinical aspects during long-term follow-up, and the necessity for multidisciplinary working are as important for general paediatricians as for pediatric gastroenterologists, surgeons and

hepatologists. The gratifying survival of increasing numbers of young people with liver disease into adult life means that it is essential also for adult practitioners with expertise in pediatric liver disease. Therefore, our research starts from the assumption that only knowledge of unexplored aspects can be an important tool for advancing into the management of hepatic disorders in children. It should be a contribute to both provide a framework to understand pathophysiology of some hepatobiliary disorders and offer analyses of their clinical-laboratory manifestations and the strategies for managing them. This project might be also useful to create specific competences related to a integrated and multidisciplinary approach, as required in pediatric liver disease. Our study concerns three areas that still present several either pathogenetic or diagnostic uncertainties, focusing on the following aspects:

1. Pathogenetic aspects of pediatric portal hypertension

- Do genetic prothrombotic risk factors play a role in pediatric portal vein thrombosis ? If so are they harmful also for other districts ?

2. Diagnostic aspects of pediatric hypertransaminasemia and unconjugated hyperbilirubinemia

- Which are the characteristics of macro-aspartate aminotransferasemia (macro-AST) in pediatric age? Are current screening tests effective? Is this a “benign” condition?
- Is electrophysiological evaluation of Crigler Najjar syndrome an helpful tool to detect Neurotoxic complications of chronic hyperbilirubinemia ?

3. Emerging complications of pediatric liver transplantation

- Is erythrocytosis a possible post-transplant complication also in liver recipients ?
- Does it exist a psychopathological risk in liver transplanted children ?

AIM

The aim of our project has been triggered by limited existing scientific evidences vs. the increasing observation of children with these problems which in clinical practice lead to frequent requests for specialist advices.

In particular, the etiology of portal vein thrombosis in children affected by portal hypertension is unknown. In this thesis, we aimed to demonstrate the possible role of genetic predisposing factors, which are still not clear in childhood. This in the perspective of individuating patients at higher risk for thrombotic events also in other districts.

Isolated increase of serum AST levels is a relatively frequent laboratoristic feature in absence of clinical symptoms. This often leads to unexpensive, time-consuming and sometimes invasive procedures. Therefore, we aimed to characterize a large series of children with cryptogenic AST levels to demonstrate the dimension and nature of macro-AST condition, and the accuracy of a simple screening test. The long term follow up was investigated as well to understand whether our previous observation that this is a benign condition is correct.

Previous studies have investigated neurological aspects of genetic chronic non hemolytic hyperbilirubinemia only in patients with severe forms of Crigler –Najjar Syndrome (type I). We aimed to assess the usefulness of neuroelectrophysiological studies also in patients with severe hyperbilirubinemia due to intermediate phenotype I/II Crigler-Najjar. This could be important to detect an early neurotoxic bilirubin effect without overt neurological damage, and to contribute for the appropriate timing of liver transplant.

The purpose of the last two projects is to study two still poorly explored aspect of pediatric liver transplantation: the investigation and characterization of erythrocytosis, a post-transplant complication which has hitherto described only in kidney recipients, and of

the psychopathological risk. We believe that to increase knowledges in the management of children undergoing liver transplantation may be extremely important because they will ultimately lead to a better quality of life. In fact it is now becoming clear that liver transplant outcome needs to be judged using a measure incorporating not only survival rates but also other features including psychological and social well being as well.

1.1. GENETIC PROTHROMBOTIC RISK FACTORS IN CHILDREN WITH PORTAL VEIN THROMBOSIS

INTRODUCTION

Portal vein thrombosis (PVT) with cavernous transformation is an important cause of portal hypertension in children (1). It is associated with gastrointestinal bleeding mainly from oesophageal and/or gastric varices (2,3). The etiology of PVT in the majority of these children is unknown. The predisposing factors can be split into three groups: direct injury to the portal vein and consequent thrombus formation, as occurs in umbilical catheterization or in omphalitis; rare congenital malformations of the vascular system associated with other cardiovascular disorders; systemic causes such as neonatal sepsis, dehydration, multiple exchange transfusions and hypercoagulable states (4).

Genetic abnormalities affecting the physiologic anticoagulant system including deficiency of protein C (PC), protein S (PS) or antithrombin (AT), have been well established as risk factors in adults (5). The recently described G1691A factor V (FVL), G20210A prothrombin (PTHR) and C677T methylenetetrahydrofolate reductase (MTHFR) mutations have also been reported as risk factors for venous thrombosis in general population (6-8) and in adult PVT patients (9-11). Prevalence of genetic abnormalities in children and adolescents with PVT has also been evaluated in few studies but the resulting data are not conclusive (12-17)

Recent reports confirm that an activating tyrosine kinase mutation, V617F JAK2 is strongly implicated in the pathogenesis of myeloproliferative disorders (MPDs) that may be associated with increased rates of thrombosis (18). Because some adult PVT patients do not suffer from an overt MPD, this mutation is considered an other important risk factor in the development of PVT independently of MPD presence.

AIM

The aim of this study was to assess the spectrum of genetic risk factors for portal vein thrombosis.

PATIENTS AND METHODS

Patients

A 1-year prospective study (from October 2007 to October 2008) was carried out at the University of Naples “Federico II”, Italy. Nineteen patients (11 males, 8 females, mean age at study \pm DS: 13.7 ± 3.7 years, range: 2.5 – 19.6 years) with portal cavernoma (mean age at diagnosis \pm DS: 5.7 ± 3.9 years) were enrolled. Portal cavernoma was confirmed by angiography and Doppler ultrasonography in 15 of them and only by Doppler ultrasonography in the remaining cases. Children with associated chronic liver and biliary diseases were excluded.

Methods

A clinical summary, with emphasis on family history of thromboembolic disease and on secondary risk factors such as umbilical vein catheterization in neonatal period, was obtained from all subjects.

Patients also underwent a complete physical examination.

Blood cell counts, routine liver tests including aspartate and alanine amino transferase activities, γ -glutamyltransferase, total and conjugate bilirubin, coagulation markers [prothrombin time (PT) and partial thromboplastin time (PTT)] were measured.

Hyperhomocysteinemia serum levels, PC, PS and AT activities were tested.

DNA was extracted from peripheral blood leukocytes according to standard protocols (18) to search G1691A FV, G20210A PTHR and V617 JAK2 mutations. C677T MTHFR polymorphism was analyzed.

The study was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects parents.

RESULTS

Doppler ultrasonography confirmed the presence of portal cavernoma in all patients.

No child had family history suggestive of thrombophilia. Twelve patients (63.1%) were found to have a history of umbilical vein catheterization in neonatal age, while the remaining 7 cases (46.9%) had not predisposing factors.

Hematemesis from rupture of oesophageal varices was the most frequent presentation [10/19 patients (52,6%)] in the first eight years of life (range 4-12 years). Splenomegaly was discovered in 8/19 patients (42.6%); only one patient showed ascites (5.2%). Overall, recurrent gastrointestinal bleeding has been observed in 14 children (73.6%) and it mostly occurred at a median interval of 1.2 year between the first and second event.

Nine patients (47.3%) were receiving beta-blockers (propranolol). Sclerotherapy or banding of varices were performed in all subjects. Seven patients (36.8%) had undergone surgical shunts: a superior mesenteric vein to intrahepatic left portal vein (Rex) shunt according to de Ville procedure in 2 of them, a portosystemic shunt in 4 and a spleno-renal shunt in the last one. Splenectomy had been made in 4 patients (21.1%).

Twelve children (63.5%) presented splenomegaly with associated thrombocytopenia ($<100.000/\mu\text{l}$) and leucopenia ($<1000/\mu\text{l}$) in 4 of them. Two patients (10.5%) had an isolated thrombocytopenia.

Liver function tests were usually normal except for occasional mild elevation in γ -glutamyltransferase in 2 patients (10.5%).

Increased levels of total bilirubin were discovered in four patients (21.1%) and were due to Gilbert Syndrome, a condition that may be associated to PVT.

Three children (31.5%) presented longer PT (mean values \pm SD: 64.1 ± 2.0 %); all remaining patients had normal coagulation values.

PC and PS activity was decreased in 8 patients (42%) [mean values \pm SD: 59.2 ± 4.5 %, range: 49.5-63.5%] and in 7 (36.8%) [mean values \pm SD: 55.1 ± 7.9 %, range: 40-64%], respectively. Serum AT activity was normal in all cases.

Heterozygous G1691A FV mutation and heterozygous G202210A PTHR mutation were found in 2 of 19 (10.5%) and in other two patients (10.5%), respectively. None carried V617F JAK2 mutation, while only one (5.2%) hyperhomocysteinemic patient was homozygous for C677T MTHFR mutation (table 1).

As shown in table 2, four (1 FV, 2 PTHR and 1 MTHFR) out of 12 patients (33.3%) with PVT and history of umbilical vein catheterization and only one (1 FV) out of 7 patients (14.3%) with idiopathic PVT had a genetic thrombophilic state.

DISCUSSION

In most of the children affected by portal hypertension, a thrombus in the portal vein with resulting cavernous transformation is the leading cause. Usually children come to attention with gastrointestinal bleeding within the first decade of life. Diagnosis of PVT needed invasive techniques such as splenic portography, but the introduction of Doppler ultrasound examination may lead to early diagnosis. Outcome of pediatric patients with PVT depends on the control of gastrointestinal bleeding from varices. Use of beta-blockers and

recent advances in the non surgical treatment of gastroesophageal varices have resulted in remarkable improvement in the clinical course of the patients.

Etiology and pathogenesis of PVT are unclear. It was initially proposed that umbilical vein catheterization in the neonatal period is responsible in almost half of the cases of newly diagnosed portal vein obstruction in children (20). Regarding umbilical vein catheterization, the predisposing factors for the development of PVT are represented by catheter misplacement, catheter dwell time over 3 days, trauma on catheter insertion and type of solution infused (20,21). It has been demonstrated that an appropriate placement of an umbilical vein catheter in neonatal age is associated with an acceptably low risk of PVT justifying the use of this procedure for fluid administration in critically ill term and preterm infants (22).

In recent years, the presence of congenital prothrombotic conditions has been considered an interesting hypothesis for the causation of PVT. Children with PVT display a reduction in PC, PS and AT activities as demonstrated by Dubuisson et al (12), but the absence of similar findings in all parents suggested that coagulation inhibitors deficiencies are not likely to be of genetic origin. Low values of PC, PS, and AT could be a reflection of an affected liver synthetic function or of their increased clearance and consume by the cavernoma itself.(12). Pinto et al (13) and Seixas et al (14) also found that hereditary PC and PS deficiency is not an etiological factor for PVT in children.

In agreement with these previous studies (12,13), PS and PC activities of our series were reduced in a relevant percentage of both groups of patients with idiopathic and post catheterism PVT (about one third of cases). PC was reduced in a lower number of patients than that reported by Chillemi et al (17) but this difference might depend on the fact that the authors did not include patients who underwent derivative surgical therapy. However may be a consequence also of other factors that at moment one cannot explain. Although G1691A FV, G20210A PTHR and C677T MTHFR mutations have recently been

reported as risk factors in general population and in adult PVT patients (6-8, 9-11), their association with PVT in pediatric age is questionable. In contrast with El-karasky (16) and Chillemi et al (17) who proved a congenital condition of thrombophilia, characterized by mutations in FV and PTHR genes in children with PVT, other studies (4,14,15) failed to demonstrate a similar correlation. No data on the role of MTHFR mutations in pediatric PVT development are available.

By large-scale epidemiological studies, prevalence of heterozygous G1691A FV mutation has been estimated to be 3-5 % of general population while heterozygous G20210A PTHR mutations are 2 to 3 % (6,7). So, in our series a congenital condition of thrombophilia namely regarding FV and PTHR was found in 5 of 19 children (26%), more frequently than the normal population, suggesting that hereditary prothrombotic disorders could be important in the development of pediatric PVT. Homozygous C677T MTHFR mutation was found in one patient (5%), but this is not relevant because of the large prevalence of homozygous state in the general population (4-26%) (8). It is interesting to note that a thrombophilic condition has been discovered mostly in the group of patients with a history of umbilical vein catheterization in the neonatal period. These data may indicate that pre-existing stress of portal vein by catheterism seems to have facilitated establishment of PVT in genetically predisposed individuals.

We extended our procoagulant screen to include V617F JAK2 analysis. This mutation leads to constitutive activation of JAK2, a cytosolic tyrosine kinase that transduces signals induced by haematopoietic growth factors including erythropoietin and stem cell proliferation. It was reported in more than 90% of patients with PV and half of patients with ET (22). Since it is well-known that PVT may be an early or presenting complication of an undiagnosed MPD, several groups have examined V617F JAK2 in adult patients with idiopathic PVT. They found that V617F JAK2 mutation occurred in these patients in a

percentage ranging from 17.2% to 45% independently of the presence of overt MPDs (18,24, 25).

The role of V617F JAK2 in development of pediatric PVT has been investigated only in one study (4). V617F JAK2 mutation screening was found to be negative in all 30 children with PVT tested. In keeping with these results, none of our patients carried V617F JAK2 mutation, but both these 2 pediatric series were extremely small.

In conclusion, a congenital prothrombotic condition observed in 26% of our children indicates that thrombophilic mutations may be involved in pediatric PVT. Preexisting stress of portal vein by catheterism might facilitate establishment of PVT in genetically predisposed individuals. We suggest that large scale studies are therefore still necessary to gain a better insight into pediatric PVT pathogenesis. Further evaluation of the V617F JAK2 mutation that has hitherto scarcely been investigated in the pediatric age needs to be included. Investigation should be extended also to JAK2 exon 12 mutations, which have most recently been shown to be possibly involved in both polycythemia vera and idiopathic erythrocytosis (26). Understanding of thrombophilic genetic factors predisposing to PVT could be helpful to identify at risk patients subgroups and prevent other possible thrombotic events also in different districts.

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TABLE 1. Coagulation disorders and genetic mutations in patients with PVT

| | Patients with normal parameters | Patients with abnormal parameters | Mean values | Range | Normal values |
|-----------------------|--|--|--------------------|--------------|------------------------|
| PT (%) | 16 | 3 (15.7%) | 64.1 ± 2.0 | 62.70-66.50 | 70-130 |
| PTT (“) | 19 | 0 | 0 | | 26- 44 |
| PC (%) | 11 | 8 (42%) | 59.2 ± 4.5 | 49.59-63.56 | 70-120 |
| PS (%) | 12 | 7 (36.8%) | 55.1 ± 7.9 | 40-64 | 70-140 |
| AT (%) | 19 | 0 | 0 | | 70-120 |
| FV (G1691A) | 17 | 2 (10.5%) Heterozygous | | | 3-5% Heterozygous* |
| PTHR (G20210A) | 17 | 2 (10.5%) Heterozygous | | | 2-3% Heterozygous** |
| MTHFR (C677T) | 18 | 1 (5.2%) Homozygous | | | 4-26% Homozygous*** |
| JAK2 (V617F) | 19 | 0 | | | 0/286 [§] |

Mutations percentage of thrombophilic factors are compared with that of general population as reported in literature:

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TABLE 2. Coagulation disorders and genetic mutations in patients with Idiopathic Portal Vein Thrombosis PVT (IPVT) and patients with post umbilical vein catheterization Portal Vein Thrombosis (PUVC - PVT)

| | IPVT patients with abnormal parameters (n=7) | Mean values | PUVC– PVT patients with abnormal parameters (n=12) | Mean values |
|---------------------------|---|------------------------|---|------------------------|
| PT (%) | 2 (28.5%) | 64.6 ± 2.7 | 1 (8.3%) | 63.2 |
| PTT (‘‘) | 0 | 0 | 0 | 0 |
| PC (%) | 4 (57.1%) | 57.8 ± 6.1 | 4 (33.3%) | 60.5 ± 2.22 |
| PS (%) | 4 (57.1%) | 56.7 ± 4.5 | 3 (25%) | 53 ± 12.12 |
| FVL (G1691A) | 1 (14.2%) Heterozygous | | 1 (8.3%) Heterozygous | 3-5% Heterozygous |
| PTHR (G20210A) | 0 | | 2 (16.6%) Heterozygous | 2-3% Heterozygous |
| MTHFR (C677T) | 0 | | 1 (8.3%) Homozygous | 4-26% Homozygous |
| JAK2 (V617F) | 0 | | 0 | 0/286 |

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Corresponding Author: Prof. Pietro Vajro, M.D.

Corresponding Author's Institution: University of Naples "Federico II", Department of Pediatrics

First Author: Maria Caropreso, M.D.

Order of Authors: Maria Caropreso, M.D.; Riccardo Campanile, M.D.; Sergio Maddaluno, M.D.; Carmelo Piscopo, M.D.; Giuseppe Castaldo, M.D.; Pietro Vajro, M.D.

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Letter to The Editor

Genetic prothrombotic risk factors in children with extrahepatic portal vein obstruction.

The predisposing role of genetic prothrombotic risk factors in pediatric extrahepatic portal vein obstruction (EHPVO) is still fragmentary and controversial. We have therefore read with great interest the article by El-hamid et al. recently appeared in the Journal (1). In a prothrombotic screening including Factor V Leiden (FVL), Prothrombin (PTHR) G20210A variant and JAK2 V617F mutations, the authors did not find any genetic variant in 30 EHPVO patients, and concluded that heritable thrombophilic factors are not involved in this condition.

We studied 19 Italian children with EHPVO (mean age \pm SD at diagnosis: 5.7 ± 3.9 years). Two patients (10.5%) had heterozygous G1691A FVL mutation, 2 others (10.5%) had heterozygous G20210A PTHR mutation. None of the children carried the JAK2 V617F mutation. Twelve/19 children (63.1%) underwent umbilical vein catheterization in the neonatal period; a genetic prothrombotic variant (1 FVL; 2 PTHR) tended to be more frequent among these 12 children (25%) rather than in the remaining 7 of the idiopathic group (1 FVL ; 14.3%).

Differently from the results of El-hamid et al. (1) and in agreement with previous studies (2, 3) our patients have a congenital condition of thrombophilia (namely regarding FVL and PTHR) more frequent than the normal population, although these data need to be confirmed on larger populations of EHPVO patients. In our series, a pre-existing stress of portal vein by catheterization seems to have facilitated establishment of EHPVO in genetically predisposed individuals. We call for large scale studies to gain a better insight into pediatric EHPVO pathogenesis, including the JAK2 V617F mutation scarcely investigated in children so far.

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*Maria Caropreso

[†]Riccardo Campanile

*Sergio Maddaluno

[§]Carmelo Piscopo

^{§+}Giuseppe Castaldo

*Pietro Vajro

*Dipartimento di Pediatria, Università di Napoli "Federico II", Napoli, Italia; [†]Chirurgia Pediatrica, AORN Santobono, Napoli, Italia; [§]CEINGE-Biotecnologie avanzate, Napoli, Italia; ⁺Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli "Federico II", Napoli, Italia.

“CLASSIC” AND “NEW” GENETIC PROTHROMBOTIC RISK FACTORS IN CHILDREN WITH PORTAL VEIN THROMBOSIS

M. Caropreso^a, S. Maddaluno^a, R. Campanile^c, M. Esposito^a, R. Piscopo^b, N. Di Cosmo^a, R. Iorio^a, G. Castaldo^b, A. Iolascon^b, P. Vajro^a

^a *Department of Pediatrics, University of Naples “Federico II”, Italy*

^b *CEINGE², University of Naples “Federico II”, Italy*

^c *Pediatric Surgery AORN Santobono, Naples, Italy*

Aims. Portal vein thrombosis (PVT) is the most common single cause of portal hypertension in children. In almost half of the cases it is associated with a history of umbilical venous catheterization (UVC), whereas in the remaining cases it is idiopathic.

The predisposing role of congenital prothrombotic risk factors is still controversial.

The aim of our study is:

- to evaluate mutations of Factor V Leiden (FVL) G1691A, Prothrombin (PTHR) G20210A, and MTHFR C677T (only in those patients with hyperhomocysteinemia).
- to investigate a new possible risk factor for PVT: JAK2 V617F. This mutation which occurs in myeloproliferative disorders (MPDs) has been found also in PVT adults patients in absence of MPDs but has hitherto not yet been investigated in paediatric PVT.

Patients and methods. We retrospectively investigated 19 children (male:female ratio = 11:8) with a median age of 13.7 ± 3.7 years (range 2.5–19.6 years) with portal cavernoma followed up at our Institutions. A complete clinical summary, with special emphasis on personal history of umbilical catheterization, admission to neonatal intensive care unit and on history of previous venous thromboembolism in the patients and their family members was obtained from all enrolled subjects. Twelve of 19 patients (63.1%) underwent UVC in the neonatal period. DNA was extracted from peripheral blood leukocytes to search G1691A FVL, G20210A PTHR and V617F JAK2 mutations in all PVT patients. C677T MTHFR mutation was evaluated only in subjects with hyperhomocysteinemia.

Results. No patient had family history of thrombophilia. Two patients (10.5%) had heterozygous G1691A FVL muta-

tion. Heterozygous G20210A PTHR mutation was detected in other 2/19 children (10.5%). None of the children carried the JAK2 V617f mutation. Only one (5.2%) patient had hyperhomocysteinemia and he presented homozygous C677T MTHFR mutation. Among patients with post-UVT portal vein thrombosis four out of 12 (33.3%) had a congenital thrombophilic state (1 FVL; 2 PTHR; 1 MTHFR). In the remaining patients with spontaneous PVT one out of 7 patients (14.3%) was heterozygous for FVL.

Conclusion. In our series a congenital condition of thrombophilia was found in 26% of children suggesting that prothrombotic disorders may be involved in the development of paediatric PVT. Preexisting stress of portal vein by catheterism seems to facilitate establishment of PVT in genetically predisposed individuals. Differently from adults the JAK2 V617F probably does not play a role in childhood portal vein thrombosis. Understanding of thrombophilic genetic factors predisposing to PVT is important for identifying at risk patients subgroups and prevent by genetic counselling other thrombotic events also in different districts.

THROMBOPHILIC GENETIC FACTORS: ARE THEY PREDISPOSING TO PORTAL VEIN THROMBOSIS IN CHILDREN?

Presenter: P. Vajro. *Università degli Studi di Napoli Federico II, Napoli, Italy.*

Co-authors: M. Caropreso¹, R. Campanile³, S. Maddaluno¹, C. Veropalumbo¹, C. Piscopo², D. Nicolina¹, I. Raffaele¹, C. Giuseppe², I. Achille². ¹*Dipartimento di Pediatria Università di Napoli Federico II, Napoli, Italy;* ²*CEINGE Università di Napoli Federico II, Napoli, Italy;* ³*Chirurgia Pediatrica AORN Santobono, Napoli, Italy.*

Background and Aim: The most common single cause of portal hypertension in children is portal vein thrombosis (PVT). It is associated with a history of umbilical venous catheterization (UVC) in almost half of the cases whereas in the remaining cases it is idiopathic. Because the role of genetic prothrombotic risk factors in pediatric PVT is still controversial we studied mutations of Factor V Leiden G1691A, Prothrombin G20210A, methylentetrahydrofolate reductase C677T, and JAK2 V617F. The latter is a mutation which plays a role in adult PVT with or without associated myeloproliferative disorders but it has not still been investigated in pediatric PVT.

Methods: We investigated 19 children (F=8) with a median age of 13.7 ± 3.7 years (range 2.5–19.6 years) with portal cavernoma followed up at our Institutions. We obtained a complete clinical history, particularly regarding umbilical catheterization, admission to neonatal intensive care unit and previous venous thromboembolism in patients and their family members. To search G1691A Factor V Leiden (FVL), G20210A Prothrombin (PTHR) and V617F Janus kinase 2 (JAK2) mutations in all PVT patients, DNA was extracted from peripheral blood leukocytes. C677T methylentetrahydrofolate reductase (MTHFR) mutation was evaluated only in subjects with hyperhomocysteinemia.

Results: Twelve of 19 patients (63.1%) underwent UVC in the neonatal period. None had family history of venous thromboembolic events. Heterozygous G1691A FVL mutation and G20210A PTHR mutation were found in 2/19 (10.5%) and 2/19 (10.5%) patients, respectively. None carried JAK2 V617F mutation, while only one (5.2%) hyperhomocysteinemic patient presented a homozygous C677T MTHR mutation. Four (1 FVL; 2 PTHR; 1 MTHFR) out of 12 (33.3%) patients with PVT and history of UVC and only one (FVL) out of 7 patients (14.3%) with idiopathic PVT had a genetic thrombophilic risk.

Conclusions: A congenital prothrombotic condition observed in 26% of our children suggests that thrombophilic mutations may be involved in pediatric PVT. Preexisting stress of portal vein by catheterism might facilitate establishment of PVT in genetically predisposed individuals.

2.1. PREVALENCE MACRO-ASPARTATE AMINOTRANSFERASEMIA (MACRO-AST) AND LONG-TERM COURSE OF IN CHILDREN

Macro-AST has become an increasingly identified but still poorly studied cause of elevated serum aspartate aminotransferase activity. It has been found in diseases such as hepatitis, various malignancies and autoimmune defects. However, this phenomenon is present in otherwise healthy subjects, in particular in pediatric age. As yet, it is not clear whether the macro-AST is persistent, nor is the pathogenesis or the prevalence of this rare condition clear.

Polyethylene glycol (PEG) precipitation is a simple test which can identify macro-AST but reference ranges of PEG-precipitable activity (%PPA) are not well established, so that confirmation by electrophoresis-the gold standard diagnostic test- is needed.

Aim of this project is therefore to investigate the prevalence, association with clinical conditions, and long-term course of macro-aspartate aminotransferase (macro-AST).

We studied for the first time a large series of healthy children with an isolated cryptogenic elevation of serum AST.

Macro-AST has been found in over one-third of our children. The cases reported suggest that is a benign phenomenon. We found that the suspicion of macro-AST should be raised not only in subjects with high AST levels but also in those with AST levels slightly higher than the normal range, after exclusion of other known causative factors of increased transaminase values. Although it is not clear what causes the formation of macro-AST, or which favours or causes

its appearance or disappearance in the blood, it may persist for years but may be also a transient phenomenon in some patients. The % precise PPA thresholds identified in the present study shows that it can be used as a screening test and that electrophoresis is helpful only to confirm positive and ambiguous results. These data may help paediatricians when they come across patients with an isolated increase of AST which does not fit the clinical condition, to avoid time-consuming, expensive and invasive procedures.

Prevalence and Long-term Course of Macro-Aspartate Aminotransferase in Children

MARIA CAROPRESO, MD, GIULIANA FORTUNATO, MD, SELVAGGIA LENTA, MD, DANIELA PALMIERI, MD, MARIANNA ESPOSITO, MD, DINO FRANCO VITALE, MD, RAFFAELE IORIO, MD, AND PIETRO VAJRO, MD

Objectives To investigate the prevalence, association with clinical conditions, and long-term course of macro-aspartate aminotransferase (macro-AST).

Study design Forty-four children with an isolated elevation of serum AST were screened for macro-AST with electrophoresis and % polyethylene glycol (PEG) precipitable activity (PPA).

Results All children were healthy, except they had elevated AST values. Seventeen children (38.6%) were macro-AST-positive. They had higher AST values than the 27 children who were macro-AST-negative ($P = .001$). Values $<67.1\%$ PPA and $>82.2\%$ PPA were associated with a very low probability of being macro-AST-positive and macro-AST-negative, respectively. Thirty-eight children underwent clinical and laboratory follow-up (mean, 4.7 ± 3.8 ; range, 1-16 years). All remained symptom-free. AST levels decreased significantly only in children who were macro-AST-negative ($P = .006$). Macroenzyme persisted in 6 of the 9 children who were macro-AST-positive after 6.0 ± 4.1 years.

Conclusions Macro-AST was present in more than one-third of children with an isolated increase of AST levels. The lack of pathological correlates in a long period argues for the benign nature of this phenomenon in childhood. We suggest that our %PPA thresholds can be used as a screening test and that electrophoresis be reserved for confirming positive screen test results and cases in which %PPA levels are of intermediate discriminant accuracy. (*J Pediatr* 2009;154:744-8)

Several enzymes form high-molecular-mass complexes (macroenzymes) by association with other plasma components or by self-polymerization. Because of their large size, they cannot be filtered by renal glomeruli and thus are retained in the plasma.¹⁻³ The condition may cause diagnostic uncertainty and very often leads to expensive and time-consuming investigations unless electrophoresis or reliable screening tests are available. This phenomenon appears to be more frequent in adults, especially those with underlying neoplastic or immune disorders.^{3,4}

Macro-aspartate aminotransferase (macro-AST) was first linked to isolated elevation in AST activity in 1978.⁵ Subsequently, this condition has been reported in patients with a wide variety of hepatic and non-hepatic diseases⁶ and in healthy people.² Reports of macro-AST in the pediatric setting are infrequent; it has been described only in a series of 4 patients⁷ and in case reports of children and adolescents in which it appears to occur mostly in healthy individuals (Table I; available at www.jpeds.com).⁸⁻¹⁶

Despite attempts to alert physicians to macro-AST,¹⁷⁻¹⁹ the prevalence and duration of this phenomenon, and the accuracy of screening tests versus electrophoresis, which is considered the diagnostic gold standard, remain obscure. The purpose of this study was to define the prevalence and course of macro-AST in children with isolated increases of serum AST levels, to determine whether it is associated with pathological conditions, to evaluate laboratory parameters in the long term, and to identify reference ranges of the screening test percent polyethylene glycol (PEG) precipitable activity (%PPA).

METHODS

Patients

Forty-four children (female/male, 20/24; mean age \pm SD, 4.2 ± 3.6 years; age range, 0.4-18 years) with persistent (>6 months) isolated high AST serum levels were referred to our department of pediatrics from 1990 to 2007. In 38 children, increased AST

From the Departments of Pediatrics (M.C., S.L., M.E., R.I., P.V.) and Biochemistry and Medical Biotechnology (G.F., D.P.), University of Naples "Federico II", Naples, Italy; CEINGE-Biotecnologie Avanzate S.p.A. r.l., Naples, Italy (G.F.); and Fondazione Salvatore Maugeri IRCCS Istituto di Campoli Telesse, Benevento, Italy (D.V.).

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Reprint requests: Prof Pietro Vajro, Department of Pediatrics, University of Naples "Federico II," Via S. Pansini 5, 80131 Naples, Italy. E-mail: vajro@unina.it.

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|-----------|----------------------------------|------|-----------------------------------|
| Macro-AST | Macro-aspartate aminotransferase | %PPA | Precipitable activity |
| PEG | Polyethylene glycol | ROC | Receiver operating characteristic |

levels were discovered unexpectedly during a well child examination that included routine laboratory testing. In the remaining 6 patients, who were from 4 families, elevated AST levels were found after the detection of isolated increase of AST serum levels in ≥ 1 of their relatives. The medical history was otherwise negative for major illnesses. None of the patients had received blood or blood-product transfusions; none consumed alcohol or had been treated with drugs that could increase liver enzyme activities during the last 6 months. One patient had undergone a liver biopsy elsewhere, the results of which were normal.

Because of the variability of upper reference levels of aminotransferase levels during childhood, we compared the serum enzyme activity of our series versus a control group of 150 subjects recruited in children undergoing minor surgery.

Laboratory Evaluation

Serum aspartate- and alanine-aminotransferase activities were determined at 37°C according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).²⁰

Patients with increased AST activity underwent routine liver tests, including alkaline phosphatase, γ -glutamyltransferase, cholinesterase, lactate dehydrogenase, total and conjugated bilirubin, and prothrombin time. Renal (blood urea, creatinine) and pancreatic functions (amylase), haemolytic disorders (blood cell count, reticulocytes, Coomb's test, haptoglobin), myopathy (creatinine kinase, aldolase), and 2 neoplastic laboratory markers (ferritin and α -fetoprotein) were also evaluated. Major causes of liver disease such as infectious hepatitis (hepatitis A virus, hepatitis B virus, hepatitis C virus, Epstein-Barr, cytomegalovirus, and toxoplasma), metabolic defects (alpha-1-antitrypsin and ceruloplasmin serum levels, urinary reducing substances and the sweat test), and autoimmunity (antinuclear, anti-smooth muscle, anti-liver-kidney microsomal antibodies and serum IgA-endomysial antibodies) were tested. Abdominal ultrasound scanning examinations and electrocardiograms were also performed.

Electrophoresis on cellulose-acetate²¹ was used as the gold standard to confirm or exclude the presence of macro-AST. The PEG screening test (precipitation with 24% PEG 6000) was performed also.^{7,22} Sera were stored at -80°C until tests were carried out. A clinical and laboratory follow-up was planned, with repetition of the PEG precipitation test and electrophoresis in patients who had a baseline diagnosis of macro-AST. The study was conducted in accordance with the Declaration of Helsinki principles. Informed consent was obtained from the patients' parents.

Statistical Analysis

Continuous variables were expressed as means \pm SD. Rate and proportions variability was expressed with the 95% CI. Analysis of 1-way variance, with Bonferroni correction, was applied to assess intergroup differences. The paired *t*-test was used to compare data from laboratory follow-up between

macro-AST value at admission and macro-AST value at the end of follow-up in the same subject.

Statistical analyses involving the %PPA or AST value variables were performed on arcs in transformed data to stabilize the variance considered the rare nature of this variable. Asymptotic 95% CI of the area under the receiver operating characteristic (ROC) curve were computed with ROCKIT software version 1.1B2 (<http://xray.bsd.uchicago.edu/krl>).²³ Probabilities and optimal threshold of macro-AST classification with %PPA were derived with a logistic model^{24,25} by using a Round-Robin algorithm for an unbiased estimation of the discrimination power. Logistic model probabilities were plotted with 99% CI bounds reflecting both the error in the estimate of the logistic parameters and the error in the estimate of the prevalence of macro-AST positivity in patients with persistent isolated elevation of serum AST. SPSS software, version 14.0 was used for standard statistical analysis.

RESULTS

Control Subjects

Mean values \pm SD and the range of serum AST and ALT were 27.8 \pm 6.3 IU/L, 14-49 IU/L, and 17.1 \pm 5.49 IU/L, 7-37 IU/L, respectively, in the 150 control children. Both AST ($P < .0001$) and ALT ($P = .005$) values tended to decrease significantly in older children (analysis of 1-way variance test). AST and ALT values were divided in 3 age subgroups: 0.0 to 2.5 years ($n = 24$), 2.6 to 7.9 years ($n = 69$), and 8.0 to 18 years ($n = 57$). AST serum levels mean values in the 3 age subgroups were 34.9 \pm 5.8 IU/L, 29.2 \pm 4.5 IU/L, and 23.1 \pm 4.7 IU/L, respectively. ALT serum levels mean values were 20.1 \pm 4.8 IU/L, 17.1 \pm 5.6 IU/L, and 15.8 \pm 5.1 IU/L, respectively. With Bonferroni correction, the decrease of AST values was statistically significant in the 3 age subgroups ($P < .001$). ALT values were reduced significantly only between the first and third age subgroups ($P = .004$). There was no sex effect.

Patients

The physical examination at study entry was unremarkable in all 44 patients. Jaundice, hepatosplenomegaly, obesity, and signs of neuromuscular and cardiac involvement were ruled out in all patients. No patient had clinical evidence of associated diseases. Of the laboratory tests, only serum AST levels were abnormal (142.8 \pm 181.0 IU/L; range, 50-1150 IU/L), with values being significantly higher in patients versus control subjects ($P < .001$). Abdominal ultrasound scanning examination and electrocardiogram results were normal in all patients.

At the first determination, 17 of 44 patients (9 girls and 8 boys) had positive results of electrophoresis test (patients who were macro-AST-positive), which corresponds to a prevalence of 38.6% (95% CI, 25.7%-53.4%; Table II). In these patients, the mean %PPA was 87.8 \pm 8.9 (range, 73-99). No patients who were macro-AST-positive belonged to the co-

Table II. Aminotransferase values, polyethylene glycol precipitable activity, and electrophoresis baseline and follow-up in patients who were macro-aminotransferase-positive

| Patient (n) | Sex | Age at entry (years) | Baseline AST (IU/L) | Baseline %PPA | Baseline electrophoresis | Follow-up (years) | Last AST (IU/L) | Last %PPA | Last electrophoresis |
|-------------|-----|----------------------|---------------------|---------------|--------------------------|-------------------|-----------------|-----------|----------------------|
| 1* | F | 3.9 | 434 | 99.0 | + | 16.0 | 74 | 84.5 | + |
| 2 | F | 4.0 | 505 | 97.0 | + | 9.0 | 242 | 94.0 | + |
| 3* | M | 8.7 | 1150 | 98.0 | + | 12.0 | 318 | 98.0 | + |
| 4 | M | 6.0 | 80 | 90.0 | + | 5.6 | 44 | 87.0 | + |
| 5 | F | 5.1 | 100 | 82.0 | + | 3.0 | 91 | 87.9 | + |
| 6 | M | 4.0 | 71 | 87.5 | + | 6.0 | 63 | 82.0 | + |
| 7 | F | 3.0 | 88 | 73.0 | + | 2.9 | 77 | 61.0 | — |
| 8 | M | 1.0 | 94 | 73.5 | + | 4.0 | 69 | 62.0 | — |
| 9* | F | 3.3 | 242 | 95.0 | + | 14.3 | 22 | 65.0 | — |
| 10 | M | 3.0 | 107 | 80.9 | + | 1.0 | 132 | NP | NP |
| 11 | F | 4.1 | 180 | 91.3 | + | 1.0 | 390 | NP | NP |
| 12 | F | 19.0 | 160 | 97.0 | + | 4.0 | 132 | NP | NP |
| 13 | F | 7.7 | 88 | 95.0 | + | 5.5 | 125 | NP | NP |
| 14 | M | 11.8 | 318 | 97.0 | + | 4.0 | 90 | NP | NP |
| 15 | F | 4.0 | 158 | 82.0 | + | NP | NP | NP | NP |
| 16 | M | 6.0 | 64 | 73.5 | + | NP | NP | NP | NP |
| 17 | M | 3.0 | 63 | 89.0 | + | NP | NP | NP | NP |

NP, Not performed; +, macro-AST-positive; —, macro-AST negative.

*First reported in reference 7.

hort of familial cases. In the remaining 27 children without electrophoretic abnormalities (patients who were macro-AST-negative), the mean %PPA was 57.7 ± 10.4 (range, 24-78), which is significantly lower than in patients who were macro-AST-positive ($P < .0001$).

The area under the ROC curve by using %PPA as a discriminator parameter between patients who were macro-AST-positive and -negative was high, 98.1% (95%CI, 91.4%-99.7%), suggesting a good level of discrimination. The sensitivity and specificity of a wide range of %PPA values is plotted in the Figure. With the projection of the sensitivity-specificity crossing point into the %PPA scale as a criterion for optimal threshold selection, the %PPA value was 73.3, which corresponds to a discrete sensitivity of 88.2% (95% CI, 65.7%-96.7%) and a discrete specificity of 88.9% (95% CI, 71.9%-96.2%). The optimal %PPA threshold (75.0) derived from the logistic model gave almost identical sensitivity and specificity values, 82.4% (95% CI, 51.1%-94.4%) and 88.9% (95% CI, 71.9%-96.2%), respectively. The Figure also shows the upper and lower 99% CI of the macro-AST probability curve derived from the logistic model. The %PPA value corresponding to a 50% macro-AST probability that defines a %PPA interval (from 67.1% to 82.2%) with an intermediate discriminating performance is shown.

Serum AST levels were significantly higher in patients who were macro-AST-positive (229.5 ± 271.3 IU/L; range, 63-1150 IU/L) than in either the 27 patients who were macro-AST-negative (88.2 ± 29.9 IU/L; range, 50-183 IU/L; $P = .001$) or control subjects ($P = .001$). A ROC curve analysis with AST serum levels as discriminator (71.7% \pm 8.3%) was significantly lower ($P = .0012$) than the %PPA ROC curve aforementioned.

Thirty-eight of the 44 patients (24/27 macro-AST-negative and 14/17 macro-AST-positive) underwent long-term clinical and routine laboratory testing follow-up (4.7 ± 3.8 years; range, 1-16 years). All patients remained in good health.

AST values decreased with time in 22 of 27 patients who were macro-AST-negative (basal value, 85.2 ± 24.5 IU/L; last value, 66.6 ± 22.2 IU/L; $P = .006$). Also, 12 of 17 patients who were macro-AST-positive showed a trend toward reduction course, although not significant (basal value, 257.7 ± 292.1 IU/L; last value, 133.2 ± 108.2 IU/L; $P = .087$).

Nine children who were macro-AST-positive underwent PEG and electrophoresis examination 6 ± 4.1 years (range, 1.2-10.5 years) after the initial macro-AST diagnosis. Six patients (patients 1-6) remained macro-AST-positive, and AST levels had decreased considerably in 4 of them (Table II). Two children (patients 7 and 8) had normalized electrophoresis and %PPA, although AST levels remained elevated. Another patient (patient 9) had normalized AST values 8 years after the first determination. AST levels remained normal in the subsequent 6 years, and both % PPA and electrophoresis were negative at the last follow-up.

DISCUSSION

Only 13 cases of pediatric macro-AST have been reported previously (Table I). Eight of these children were healthy.^{6,7,9,11,12} In the remaining 5 patients (4 adolescents), macro-AST was discovered during the investigation of lower back pain,^{15,16} seizures,¹³ and inflammatory bowel disease.^{8,10} The macro-AST phenomenon could have been related to immune processes in the latter 2 patients only.^{8,10} Data about the prevalence of macro-AST and its clinical course are scarce

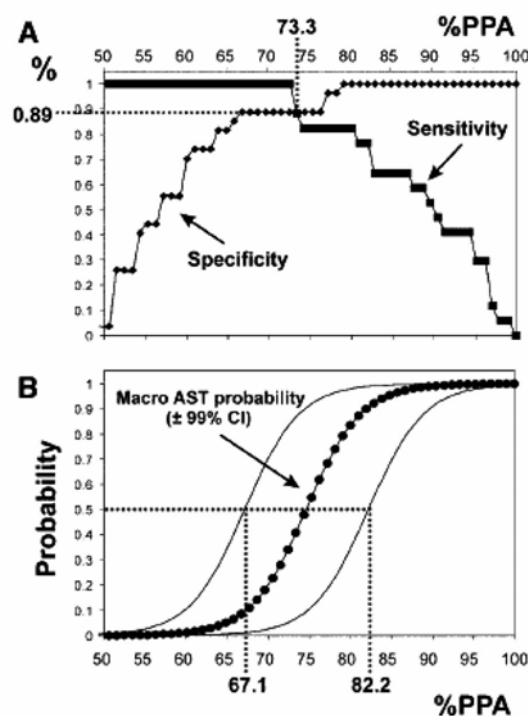


Figure. A, Sensitivity (square) and specificity (diamond) plot versus %PPA threshold values showing the crossing point figures marked (89% sensitivity and specificity at 73.3 %PPA). B, Probability of macro-AST as a function of %PPA derived with the logistic model. %PPA thresholds corresponding to a probability of 50% are indicated for both 99% CI curves (67.7-82.2). %PPA values less than or higher than this interval relate to a very low and very high probability of macro-AST, respectively, and the middle has figures with intermediate values.

for all age groups. Because of the relatively large number of children with isolated elevation of AST values enrolled in this study, we were able to calculate the prevalence of macro-AST (17/44; 38.6%) in children with abnormal serum AST levels with reasonable accuracy.

At the first evaluation, AST levels were significantly higher in patients who were macro-AST-positive than in either patients who were macro-AST-negative or control subjects. We previously reported AST values at least 4-fold times the upper limit of the reference range in patients who were macro-AST-positive.⁷ This study shows that even AST values slightly exceeding the upper reference range, as seen in the follow-up of case 4, do not exclude the diagnosis of macro-AST.

No long-term follow-up data for macro-AST have been reported previously. In our study, follow-up spanned from 1 to 16 years, with a mean of 4.7 years.

In most of our macro-AST-negative cases, serum AST values tended either to remain stable or to decrease with time,

so that the mean AST activity decreased significantly during follow-up. We do not know why AST levels were high in patients who were macro-AST-negative, because most causes of hyperaminotransferasemia were ruled out with tests performed at entry and, in several cases, also during follow-up. The results of a liver biopsy performed in 1 case were normal. All the patients who were macro-AST-negative were clinically healthy, and in most cases, AST values were far from the low standard deviations of healthy control subjects that can be considered within the tails of the normal distribution. Although electrophoresis is the gold standard method for macroenzyme detection and is a sensitive diagnostic tool²¹ and the macro-AST positivity values were borderline (Table II), we cannot exclude the possibility that they might represent false-negative results.

In our macro-AST positive group, AST levels with time tended to differ in subjects. The macro-AST phenomenon was transient in the 2 patients with the lowest %PPA values (73.0% and 73.5%; patients 7 and 8, respectively), which were within the intermediate probability area of the ROC curve, and in patient 9. We are unable to explain the persistence of elevated AST in patients 7 and 8. For patient 9, AST normalization has been reported in a case with fluctuating AST values, although repeated diagnostic tests for macro-AST were not available.²⁶

In a large study of macro-AST conducted in adults, the condition was associated with several diseases.⁶ However, patients had a high serum aspartate to alanine aminotransferase ratio, and the study included a large number of subjects with liver and biliary diseases. It is possible that the condition affecting these adults does not reflect the true condition of macro-AST (isolated increase of serum AST), which may explain the lower prevalence of the condition in adults⁶ than in children (13.1% versus 38.6% in our series).

We found no relationship between macro-AST and illness at diagnosis or during follow-up. With the exception of the previously reported association with inflammatory bowel diseases,^{8,10} our data suggest that macro-AST may be considered a benign condition in children and adolescents and is not a harbinger of other disorders.

The pathogenesis of macro-AST is unknown, but could involve an individual genetic predisposition to make macro-complexes or an abnormal response of the immune system, as in the case of inflammatory bowel diseases.⁴ A study of first-degree relatives of patients with macro-AST failed to reveal a familial component.⁷ Our series includes 4 families with an unexplained increase of AST in >1 member. None of them had macro-AST. The PEG precipitation technique is used to detect macro-AST, but reference ranges have yet to be defined for both adults and children. Davidson and Watson¹ proposed several reference ranges of precipitable activity for several commonly measured enzymes in plasma, but suggested their data be tested on larger samples of patients. Their 3 adults with macro-AST and associated organic diseases had elevated values of %PPA (range, 71%-97%). We identified 2 %PPA thresholds, 67.1% and 82.2%. Values less than this

interval indicate a very low probability of being macro-AST-positive, whereas values higher than this interval indicate a very high probability of being macro-AST-positive. To optimize the procedure for macro-AST screening in large population samples, we suggest electrophoretic analysis be reserved for confirming positive results on screening tests and cases in which %PPA are of intermediate discriminant accuracy. Electrophoresis is time-consuming, difficult to set up, and requires special equipment, whereas the PEG screening technique (evaluating AST values before and after challenging patients' sera with PEG) is a simple, practical tool easily available in all laboratories and is used for the screening of all macroenzymes (lactate dehydrogenase, creatine kinase, alkaline phosphatase, γ -glutamyltransferase). The PEG is also considerably cheaper than electrophoresis. However, recently it has been confirmed that the PEG screening precipitation technique still needs standardization and defined reference ranges.²⁷

In conclusion, we report the prevalence of macro-AST in a population of otherwise healthy children and adolescents with an isolated cryptogenic increase of serum AST. During a long follow-up period, no associated conditions developed in any child. Although we cannot exclude the possibility that, in some cases, the macro-complex formation might have some clinical relevance, we suggest that, in the pediatric age, it be considered most likely benign. Although considerably high AST values in an individual with no other laboratory test result abnormalities will alert the physician to the possibility of macro-AST, even AST levels slightly higher than the reference range should be investigated further. In addition, macro-AST may be a transient phenomenon in some patients. Within the thresholds reported, the PEG test is a reliable tool that may be used for screening large populations. This may avoid time-consuming, expensive, and sometimes invasive procedures.

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Table 1. Reports of pediatric macro-aminotransferase

| Author | Reference | Patients (n) | Age (years) | Sex (M/F) | Baseline AST (IU/L) | Follow-up of AST values | Associated condition |
|----------------------------|-----------|--------------|-------------|-----------|---------------------|--|-------------------------------|
| Perrault et al 1990 | 8 | 1 | 16 | F | 150 | Not reported; persistence of high AST | Crohn's disease |
| Moriyama et al 1990 | 6 | 1 | 6 | F | >100 | Not reported | Not reported |
| Vajro et al 1992 | 9 | 1 | 4 | F | 434 | 1 year; persistence of high AST (380 IU/L) | Healthy |
| Tajiri et al 1992 | 10 | 1 | 14 | M | 128 | 3 years; normalization of AST paralleling ulcerative colitis after 2 years | Ulcerative colitis |
| Matama et al 1993 | 11 | 1 | 17 | F | 152 | 4 years; persistence of high AST (124 IU/L) | Healthy |
| Fortunato et al 1998 | 7 | 4* | 6-10 | 2M/2F | 434; 1150; 242; 233 | 0.5-6.0 years; persistence of high AST (627; 1125; 262; 243 IU/L) | Healthy |
| Agbo-Kpati et al 1999 | 12 | 1 | 10 | F | 145 | 6 months; persistence of high AST (132 IU/L) | Healthy |
| Monfort-Gouraud et al 1999 | 13 | 1 | 16 | F | 524 | 6 months; persistence of high AST (524 IU/L) | Seizures |
| Wiltshire et al 2004 | 14 | 1 | 3.5 | F | range, 714-1046 | Not reported | Healthy |
| Cabrera-Abreu et al 2008 | 15 | 1 | 6 | M | 113 | 1 month; persistence of high AST (100 IU/L) | Lower back pain, constipation |
| Lord et al 2008 | 16 | 1 | 15 | F | 407 | 1 month; persistence of high AST (342 IU/L) | Lower back pain |

F, Female; M, male.

*One of the 4 cases was described as "Case Report" in Vajro et al 1992.⁹

PREVALENCE AND LONG-TERM FOLLOW-UP OF MACRO-ASPARTATE AMINOTRANSFERASE (MACRO-AST) IN CHILDREN
VAJRO P¹, FORTUNATO G², CAROPRESO M¹, IORIO R¹, VEGNENTE A¹,
LENTA S¹, DI COSMO N¹, CANDIELLO A¹, ESPOSITO M¹, CIRILLO F¹
(1) Dept of Pediatrics -University "Federico II", Naples, ITALY. (2) Dept of
Biochemistry and Medical Biotechnology-University "Federico II", Naples, ITALY.

Aim: To investigate prevalence, association with other clinical conditions, and long-term follow-up of macro-AST, a still poorly characterized cause of isolated elevation of serum AST.

Methods: From 1990 to 2006 we observed 44 consecutive children (F/M 20/24; mean age $4.2 \pm SD 3.6$; range 0.4-19 yrs) with persistent (>6 mos) isolated increase of serum AST levels (hyper-AST) as compared with n.v. of 150 age and sex matched controls. Physical examination and other routine laboratory tests were normal in all. The main causes of hypertransaminasemia were excluded. Macro-AST presence was screened by polyethylene glycol (PEG) precipitable activity (% PPA; n.v. <73%) and confirmed by electrophoresis. 38 pts underwent a long term clinical and laboratory follow up (F.U.) with repeated AST measurements.

Results: The table shows AST values at first evaluation & macro-AST status:

| | n | Basal AST (IU/L) mean \pm SD & range | p |
|--------------------------|-----|--|---------------|
| 1) Controls | 150 | 27.8 ± 6.3 (14-49) | |
| 2) Hyper-AST (total pts) | 44 | 121.9 ± 95.7 (50-505) | 2 vs 1 <0.001 |
| 3) Hyper-AST Macro [] | 27 | 88.2 ± 29.9 (50-183) | |
| 4) Hyper-AST Macro [] | 17 | 175.4 ± 135.0 (53-505) | 4 vs 3 0.02 |

During F.U. (mean $4.7 \pm SD 3.9$; range 0.3-16 yrs) all the 17 pts with macro-AST had a stable clinical picture. In 9 of them repeated macro-AST evaluation showed that 3/9 had unchanged AST values, %PPA and electrophoresis; 2/9 had a considerable decrease of AST levels in spite of unmodified % PPA; 1/9 normalized AST values, %PPA and electrophoresis; 1/9 still had macro-AST despite a progressive reduction of AST levels; 2/9 resulted negative for both macro-AST tests in spite of persistently high isolated AST serum levels.

Summary and conclusions: In this series of children with isolated increase of AST serum levels, macro-AST is present in more than one third of cases. Our data suggest that it is a clinically benign phenomenon. It may persist for several years or disappear independently from serum AST values. Although macro-AST is generally characterized by high serum enzyme activity, it must be taken into account also in cases with slightly abnormal AST values.

LONG-TERM COURSE OF MACRO-ASPARTATE AMINOTRANSFERASE (MACRO-AST) IN CHILDREN

M. Caropreso^a, G. Fortunato^{b,c}, N. Di Cosmo^a, S. Lenta^a, M. Esposito^a, S. Maddaluno^a, I. De Napoli^a, R. Iorio^a, P. Vajro^a

^a *Departments of Pediatrics University of Naples "Federico II", Italy*

^b *Biochemistry and Medical Biotechnology, University of Naples "Federico II", Italy*

^c *CEINGE-Advanced Biotechnologies scarl, Naples, Italy*

Objectives. To investigate prevalence, association with clinical conditions, and long-term follow-up of paediatric macro-aspartate aminotransferase (macro-AST), a still poorly characterized cause of isolated elevation of serum AST.

Methods. From 1990 to 2006 we observed 44 consecutive children with persistent isolated increase of serum AST, as compared with normal values of 150 age- and gender-matched controls. Macro-AST was studied by electrophoresis (gold standard) and by % polyethylene glycol (PEG) precipitable activity (%PPA). A clinical and laboratory follow-up was planned.

Results. At entry all patients were clinically healthy; their AST values were significantly higher than those of controls (142.8 ± 181.0 vs. 27.8 ± 6.3 IU/l; $p < 0.001$). Seventeen patients (38.6%) were macro-AST positive. Their AST values were higher than those of the macro-AST negative patients (229.5 ± 271.3 IU/l vs. 88.2 ± 29.9 IU/l; $p = 0.001$). PEG test was highly suggestive of macro-AST when % PPA was >78.5 (sensitivity 82%, specificity 100%), with a grey area in the 70–78.5% range. Thirty-eight patients (14 with macro-AST) underwent a long-term clinical and laboratory follow-up (4.7 ± 3.8 , range 1–16 years). All remained symptoms-free, and in both macro-AST negative and positive groups repeated AST measurements showed a decrease in enzyme activity, which was statistically significant only in the former one ($p = 0.006$). In nine macro-AST positive patients further macro-AST evaluation after 6 ± 4.1 years (range 1.2–10.5 years) showed that the phenomenon persisted only in six.

Conclusions. In this series of children with isolated increase of AST serum levels, macro-AST is present in more than one-third of cases. Our data suggest that it is a clinically benign phenomenon which may have a transient course. Although macro-AST is generally characterized by high serum enzyme activity, it must be taken into account also in cases with slightly abnormal AST values. The PPA cut off of 78.5% consents to identify the presence of the macroenzyme with good sensitivity and excellent specificity. For values of % PPA ranging from 70 to 78.5, it is necessary to confirm PEG results with electrophoresis.

2.2. CLINICAL UTILITY OF ELECTROPHYSIOLOGIC EVALUATION IN CRIGLER-NAJJAR SYNDROME

Crigler Najjar (CN) syndrome is an extremely heterogeneous condition with high grade increase of neurotoxic unconjugated bilirubin. Severe forms (type I CN) need liver transplantation (OLT) to survive. Sometimes clinical picture of mild forms (type II CN) overlaps with those of severe forms leading to an intermediate variety (type I/II CN) that represents a diagnostic and therapeutic challenge. In fact, asymptomatic patients with borderline hyperbilirubinemia values may suddenly present with severe neurological damage during adolescence or early adulthood. In these cases neurophysiologic features have not been characterized. So far, it is therefore not clear how to monitor long-term neurological aspects of CN patients in cases with raised bilirubin levels and not yet overt neurological damage to establish the appropriate timing of OLT.

In the series studied by us only electroencephalogram (EEG) and visual evoked potentials (VEPs) seemed to be correlated with high bilirubin levels. Since VEPs are a quantitative test, we propose this test as a useful tool to monitor the neurotoxic effects of hyperbilirubinemia. Differently from brainstem auditory (BAEPs) and motor (MEPs) evoked potentials, EEG and VEPs contribute to detect and monitor bilirubin neurotoxic effects and may play a decisional role alerting physicians for OLT evaluation in those cases of severe hyperbilirubinemia without clinical findings of overt neurologic damage.

Clinical Utility of Electrophysiological Evaluation in Crigler-Najjar Syndrome

Author

A. Perretti¹, G. Crispino², L. Marcantonio¹, S. Lenta², M. Caropreso², F. Manganeli¹, S. Scianguetta³, R. Iorio², A. Iolascon³, P. Vajro²

Affiliation

¹ Department of Neurological Sciences, University of Naples Federico II, Naples, Italy

² Department of Pediatrics, University of Naples Federico II, Naples, Italy

³ CEINGE – Advanced Biotechnology, University of Naples Federico II, Naples, Italy

Key words

- Crigler-Najjar syndrome
- electrophysiological features
- UGT1A1 gene

Abstract

We evaluated the neurological and neurophysiological features in ten patients with genetically characterized Crigler-Najjar (CN) syndrome: four with typical type I CN had undergone orthotopic liver transplantation (OLT); six had type II CN, and three of them developed severe hyperbilirubinemia with a limited response to phenobarbital leading to an intermediate phenotype I/II. Clinical neurological and multimodal electrophysiological evaluations [electroencephalogram (EEG), visual (VEPs), motor (MEPs) and brainstem auditory (BAEPs) evoked potentials] were performed. Neurological examinations showed mild hand tremor in four patients (one pre-OLT and one

post-OLT type I, two type I/II). EEG revealed high voltage paroxysmal discharges in four patients (three type I/II, and one type I with a marked improvement after OLT). VEPs showed P100 wave increased latency in five patients (three type I, and two type I/II considered for OLT evaluation). MEPs showed prolonged central motor conduction time in five patients (two type I; one type I/II; two type II). Only EEG and VEPs findings showed a correlation with high bilirubin levels. BAEPs were normal. In conclusion, VEPs and EEG contribute to identify and monitor bilirubin neurotoxic effects, and may play a decisional role in some cases of severe hyperbilirubinemia without overt neurologic damage.

Introduction

Crigler-Najjar (CN) syndrome is a rare autosomal recessive inborn error of bilirubin (BR) metabolism, which is responsible for a chronic elevation of serum levels of neurotoxic unconjugated BR. Depending on residual activity of bilirubin UDP-glucuronyl-transferase (UGT), levels of serum bilirubin and response to phenobarbital (PB) treatment, the syndrome may present in a form with a severe (type I CN; BR values usually > 20 mg/dL) or a mild (type II CN; BR values usually < 20 mg/dL) clinical picture [2,5]. In spite of this classification, type II CN appears clinically heterogeneous making it at times difficult to classify some cases [11,13]. In several type II CN patients with an intermediate response to phenobarbital, severe hyperbilirubinemia could persist especially during acute illnesses. This leads to a clinical picture overlapping that of type I CN (overlap type I/II CN) which may require several additional medical treatment (e.g., phototherapy, cholestyramine, plasmapheresis and albumin infusion). In some of these cases associated

mutations for Gilbert syndrome and/or hormonal changes at puberty may be an additional cause [4].

Although untreated CN patients with marked hyperbilirubinemia generally present early with severe clinical neurological damage [18,22], some adult cases without manifest clinical neurological abnormalities have been described [6,7]. Conversely, seemingly asymptomatic patients with mild hyperbilirubinemia may suddenly present with severe neurological damage during adolescence or early adulthood [3]. In these cases neurophysiological features, however, have not been studied. In children and adolescents neurophysiological investigations of CN syndrome have been performed only in type I CN [15,19]. Because it is important to better understand how to avoid neurological sequelae of chronic hyperbilirubinemia at all ages, we report the clinical and neurophysiological features in a group of genetically characterized adolescents and young adults with CN syndrome.

The need for liver transplantation is usually driven by the clinical and biochemical back-

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Correspondence

Prof. P. Vajro
Dipartimento di Pediatria
Università di Napoli
"Federico II"
Via S. Pansini 5
80131 Napoli
Italy
Tel.: +39/081/746 26 77
Fax: +39/081/546 98 11
vajro@unina.it

ground. However, pending stricter criteria as to the right time of transplantation, our neurophysiological data might give a further contribution in the management of some cases of severe hyperbilirubinemia without overt neurological damage.

Patients and Methods

▼

Patients

We studied ten CN patients (M:F=7:3; mean age: 18.0 ± 4.1 years, range 14.0–29.6, clinical follow-up: 9.0 ± 5.7 years). As shown in **Table 1**, four were type I, three overlap type I/II, and three were type II CN patients. Most received a diagnosis of Crigler-Najjar syndrome in infancy and were followed during adolescence and young adulthood when the present neurophysiological study was performed. Three patients with CN II came to our attention during adolescence.

Patients 1–7 had undergone nocturnal phototherapy averaging 9–12 hours daily for several years. However, nocturnal phototherapy could not avoid bilirubin increases with age and it was a considerable source of social inconvenience. All four patients with type I CN underwent OLT at the age of 10.2, 7.0, 4.6 and 5.7 years respectively (time elapsed from OLT at evaluation: 11.05 ± 2.9 years, range 7.2–13.6) with subsequent normalization of bilirubin levels (range of highest bilirubin levels before OLT: 28.0–35.1; after OLT <1.0 mg/dL), except in one patient who received the organ from a subject with Gilbert syndrome [22]. As shown in **Table 1**, their bilirubin/albumin molar ratios pre-OLT were $>$ the “safe” value of 0.7 but always <1 (“albumin saturation”) [20]. These patients are chronically treated with immunosuppressive therapy (cyclosporine or tacrolimus) and have not shown related-major complications during follow-up, including possible signs of neurotoxicity correlated to calcineurin inhibitors.

Three other patients under PB therapy presented with an overlap type I/II CN picture, and despite an initial response to PB treatment with a decrease of BR levels of approximately 30%, their BR progressively rose, especially around the time of puberty and became close to those of type I CN. Because of the increasing bilirubin levels they required the addition of cholestyramine treatment and phototherapy. Repeated plasmapheresis procedures with albumin infusion to improve bilirubin/albumin molar ratios were necessary during acute hyperbilirubinemia exacerbations, especially during episodes of acute illnesses or poor adherence to phototherapy. These measures were undertaken to avoid dangerous values of hyperbilirubinemia exceeding the potential threshold beyond which neurotoxicity is likely to develop. Highest BR levels and their corresponding bilirubin/albumin molar ratios were 30.3, 26.3, 20.7 mg/dL and 0.67, 0.63, 0.52 μ mol, respectively (**Table 1**). These patients, in particular the first two who had bilirubin/albumin molar ratios often near to the critical value of 0.7, were felt by the medical team to be at high risk for sudden irreversible neurological deterioration.

Three patients had classic type II CN: one whose highest BR levels was 4.0 mg/dL was untreated, two others were under chronic treatment with phenobarbital (therapeutic range = 10–30 ng/mL) for cosmetic reasons (highest BR levels before PB: 8.0 and 9.0 mg/dL, highest BR levels after PB: 4.0 and 5.0 mg/dL, respectively) (**Fig. 1**). Bilirubin/albumin molar ratios were always <0.25 (**Table 1**).

Methods

All patients underwent a molecular study of mutations related to the UGT1A1 gene and its promoter (TATA box) for the detection of concomitant Gilbert syndrome, clinical, and multimodal electrophysiological evaluation [electroencephalogram (EEG), visual (VEPs) and brainstem auditory (BAEPs) evoked potentials]; nine patients underwent motor evoked potentials (MEPs) at the upper and lower limbs in six and at the upper limbs in three. Five patients underwent follow-up EEG, VEPs and BAEPs.

Molecular studies of UGT1A1 gene

Anticoagulated (EDTA-treated) blood was obtained from all patients. All five exons of UGT1A1 and the flanking intron sequences were amplified by polymerase chain reaction (PCR). The amplified products were isolated by electrophoresis on a 1% agarose gel and then purified using the QIAamp purification kit (Qiagen) and the nucleotide sequence was determined by direct sequencing. The analysis of the A(TA)_n TAA motif in the promoter region of the UGT1A1 gene was performed by means of PCR. The product was resolved on 6% denaturing polyacrylamide gel and silver staining [17].

Clinical evaluation

Anamnestic data showed that none of the patients had gross neurological sequelae of hyperbilirubinemia such as seizures, cognitive decline, pyramidal, extrapyramidal, cerebellar dysfunction and hearing loss.

At the time of neurophysiological study all patients underwent a complete physical and neurological examination.

Neurophysiological evaluations

Electroencephalogram (EEG)

EEG recordings were performed according to the 10–20 International System with 13 bipolar montages (Fp2–C4, Fp1–C3, C4–O2, C3–O1, T4–O2, T3–O1, T4–C4, T3–C3, Fp2–T4, Fp1–T3, C4–C3, T4–T3, O2–O1). The EEG recordings were obtained by Micromed electroencephalograph (System Plus Software) with the electrode impedance below 5 K Ω m. EEG was carried out during basal conditions and after hyperventilation and photic stimulation. A follow-up study was performed in five patients.

Evoked Potentials

VEPs, BAEPs and MEPs were recorded by a Keypoint electromyograph (Medtronic, Denmark) via surface disk electrodes, 11 mm in diameter (Medtronic 13L29), filled with conductive electrode gel with impedance kept below 5 K Ω m.

Motor evoked potentials (MEPs)

A magnetic stimulator (Magstim 200, Magstim Company, Ltd, Whitland, UK), producing a monophasic transient magnetic field of 2.0 Tesla at maximum field strength, was used for transcranial magnetic stimulation (TMS) of the motor cortex. The circular coil (outer diameter 14 cm) was placed tangentially on the skull, centered over the vertex for upper limb and 2 cm anteriorly to the vertex for the lower limb; an anticlockwise current circulation in the coil was used to stimulate the left hemisphere. For recording muscle evoked potentials (MEPs), the transcranial magnetic stimulation intensity used was 20% above motor threshold (the threshold was defined as the stimulus intensity required to produce a MEP at rest of 50–100 μ V amplitude occurring in 50% of 10–20 consecutive stimuli). MEPs were obtained in contracted right abductor pollicis brevis muscle (APB) at the

Table 1 Clinical, genetic, laboratory and neurophysiological findings of the 10 patients with CN syndrome

| Case no. | Age at last evaluation (years)/gender | Clinical phenotype | Age at OLT (years) | Genetic analysis | Highest serum BR level and BR/Alb ratio | Clinical examination | EEG | MEP | VEP | BAEP |
|----------|---------------------------------------|--------------------|--------------------|---|---|--|------------------------------|----------------------------------|------------|--------|
| 1 | 17.4/M | CN I | 10.2 | c.878_890 del ins A-W461R | pre-OLT or MT 28.0 (0.80) post-OLT during MT 0.7 (0.02) | A (rest/postural hand tremor pre-OLT) N (post-OLT) | A/A (pre-OLT) A/N (post-OLT) | NP A (upper limb) A (lower limb) | NP N/N | NP N/N |
| 2 | 19.8/M | CN I | 7.0 | c.879_890 del ins A-c.879_890 del ins A | 35.1 (0.90) | N | N (post-OLT) | A (RE, LE) | A (RE, LE) | N |
| 3 | 18.2/M | CN I | 4.6 | c.879_890 del ins A-c.879_890 del ins A | 33.0 (0.80) | N | N (post-OLT) | N | A (LE) | N |
| 4 | 16.3/F | CN I | 5.7 | c.717_718 del AG-c.717_718 del AG | 34.6 (0.86) | A (postural hand tremor) | N (post-OLT) | N | A (LE) | N |
| 5 | 18.5/M | CN II | | W461R-W461R | 23.5 (0.67) | A (action hand tremor) | A/A/A/A | A/A/A | N/N | N/N |
| 6 | 29.6/F | CN II | | R209W | 18.0 (0.47) | N | A/N | A (upper limb) | N/N | N/N |
| 7 | 14.0/M | CN II | | N279Y + TA6/TA7 | 15.6 (0.38) | A (rest/postural hand tremor) | A/A | N | N | N |
| 8 | 17.5/M | CN II | | V225G-G377V + TA6/TA7 | 4.0 (0.10) | N | N | A (lower limb) | N | N |
| 9 | 19.3/M | CN II | | c.878_890 del ins A + TA6/TA7 | 8.0 (0.19) | N | N | A (lower limb) | N | N |
| 10 | 17.0/F | CN II | | R403C + TA6/TA7 | 9.0 (0.23) | N | N/N | A (lower limb) | N/N | N/N |

OLT = orthotopic liver transplantation; BR = bilirubin (mg/dL); BR/Alb = bilirubin/albumin molar ratio; MT = medical therapies in addition to phenobarbital; EEG = electroencephalogram; VEP = visual evoked potentials; MEP = motor evoked potentials, BAEP = brainstem auditory evoked potentials; * post-OLT Gilbert's

N = normal; A = abnormal; RE = right eye; LE = left eye; / = repeated test; NP = not performed

All data refer to post-OLT or post-MT, except for patient no. 1 whose clinical evaluation and EEG were studied before and after OLT

upper limb and in contracted left abductor hallucis muscle (AH) at the lower limb (active electrode placed on the muscle belly and reference electrode on tendon). During recording active muscular contraction (20% of maximum voluntary contraction) was maintained with the help of auditory and visual electromyographic feedback. Filter bandpass was 10–2000 Hz. The shortest onset latency and the largest peak-to-peak amplitude of four consecutive MEPs were measured. Radicular responses were recorded in the abductor pollicis brevis muscle at rest, stimulating cervical roots by positioning the magnetic coil in the midline over the lower cervical vertebrae to obtain the longest latency. Central motor conduction time was obtained from the latency difference between MEPs and radicular responses for APB muscle, and using the F technique for AH muscle. MEPs were performed according to Recommendations for the Practice of Clinical Neurophysiology (Guidelines of the International Federation of Clinical Neurophysiology) [14].

Visual evoked potentials (VEPs)

VEPs were recorded at Oz (active electrode) and Fpz (reference electrode) of the International 10–20 system stimulating each eye, in turn, with a checkerboard reversal pattern, at a frequency of 1.5 Hz. The visual angle subtended by the single checks and by the whole monitor measured 30 minutes and 18 degrees, respectively. Two consecutive sets of 100 responses were averaged and superimposed. The filter bandpass was 0.5–100 Hz. The P100 latency of the negative-positive-negative complex (N75-P100-N145) was measured, and the amplitude was calculated peak to peak from the N75 to the P100 wave.

Brainstem auditory evoked potentials (BAEPs)

BAEPs were recorded from the vertex (Cz) referred to the earlobes ipsilateral and contralateral to the stimulation side in a soundproof room. Each ear, in turn, was stimulated using condensant clicks resulting from a 100 μ s monophasic square electrical pulse, at 65 dB above the individual click-hearing perception threshold, at a stimulation rate of 11 Hz; the contralateral ear was stimulated by a white masking noise. Two consecutive sets of 2000 responses were averaged and superimposed for each ear with a bandpass of 100–2000 Hz. The latency of the first five positive peaks and the I-III, I-V, and III-V interpeak latencies were measured. A follow-up study was performed in five patients.

Motor, visual and brainstem auditory evoked potential findings were compared with age-matched controls data obtained by the same techniques in our laboratory. All of the latency values deviated by more than 2.5 SD from the mean control value were considered abnormal, as were amplitude values below the minimum control value.

Results

Genetic analysis in type I CN patients showed small deletions with involvement of both alleles (100% cases) whereas type II and overlap type I/II patients showed missense mutations (83% cases) on both alleles (34% cases) or one allele (66% cases). In particular, subjects affected with CN-II were double heterozygotes for a missense mutation in one allele and a TA7 promoter polymorphism in the other. This situation causes a severe reduction of UGT1 enzymatic activity.

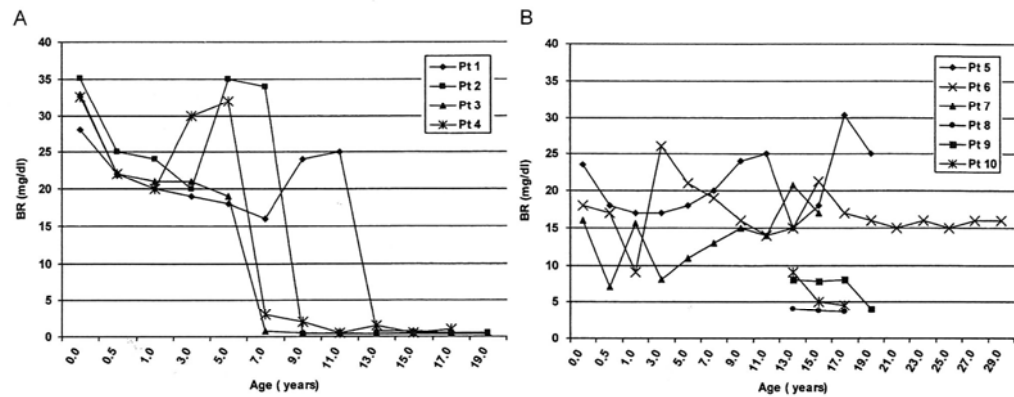


Fig. 1 Panel A shows bilirubin (BR) values and course of four CN type I patients (#1, 2, 3, 4). Note the dramatic decrease of BR values after OLT at age of 10.2, 7.0, 4.6, 5.7 years, respectively. Panel B shows bilirubin values and course of three CN overlap I/II (#5, 6, 7) and three CN type II (#8, 9, 10) patients. Bilirubin serum levels show a fluctuating pattern during treatment with plasmapheresis, phenobarbital, phototherapy, cholestyramine and in relation to intercurrent acute illnesses.

Clinically, all patients were healthy except for evidence of jaundice of variable grade depending on their serum bilirubin levels. Standard liver function tests other than bilirubin were within normal limits, except for serum gamma glutamyltranspeptidase and alkaline phosphatase levels which were elevated in those who were under phenobarbital treatment.

As illustrated in **Table 1**, clinical neurological examination showed mild hand tremor in four patients: one type I CN pre-OLT (with normalization after OLT) and two overlap type I/II patients who had a rest/postural tremor and an action tremor; one type I post-OLT patient showed a postural tremor. Developmental aspects were not evaluated in depth. However, we knew that seven patients who were still students had a sufficient school performance and three who were already employed served as general office clerks without particular difficulties.

Four out of ten patients showed EEG abnormalities consisting in high voltage paroxysmal discharges (three overlap type I/II and one type I). At follow-up of four patients, in one type II patient the EEG remained normal; in two overlap type I/II patients EEG abnormalities were confirmed, whereas the test became normal after 2 years in the third one. In a type I CN patient the EEG improved 1 year after liver transplantation and was found completely normal at 7 years follow-up.

TMS MEP findings showed a prolonged central motor conduction time in five out of nine studied patients (two type I, one overlap type I/II, two type II patients), all clinically asymptomatic (**Fig. 2**).

VEPs showed an increased latency of P100 wave in five out of ten patients (three type I and two overlap type I/II patients) (**Fig. 3**). None of them suffered from visual impairments. Two out of the five patients who underwent follow-up showed a significant worsening of P100 latency prolongation coinciding with a higher bilirubin level for which they were considered for liver transplantation evaluation. Both had a clinical phenotype of CN I/II, and at the present time one of them (patient 5, **Table 1**) is on the active waiting list for liver transplantation. BAEPs did not show central abnormalities at the basal and follow-up examinations.

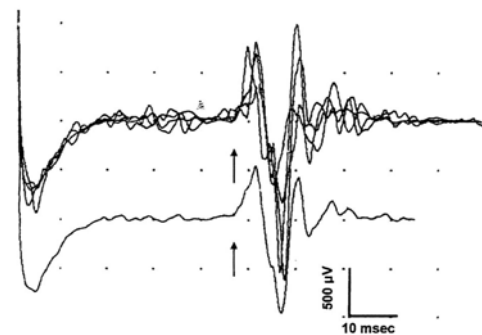


Fig. 2 Transcranial magnetic stimulation motor evoked potentials (MEP) recorded in abductor hallucis muscle in patient #9: note a prolonged MEP latency (46.8 msec; maximum normal value 42.8 msec).

Discussion

As expected from a literature review, genetic characterization of our patients showed that the most common mutations occurring in type I CN (marked hyperbilirubinemia unresponsive to PB) were non-sense mutations; conversely missense mutations were more commonly found in patients with type II and overlap I/II CN syndrome (variable degrees of hyperbilirubinemia fully or partially responsive to PB, respectively). Four patients had clinical evidence of neurological involvement (mild hand tremor). Interestingly, only one of the type I CN patients had mild neurological clinical abnormalities after liver transplantation. This may suggest the possible reversibility of neurologic injury after a prolonged exposure to marked hyperbilirubinemia. This was confirmed by the finding that all the four transplanted patients with type I CN syndrome had a normal EEG after transplantation. Probably this was an effect of the rather early operation (mean age at intervention 6.8 years) which interrupted in time the exposure to severe hyperbiliru-

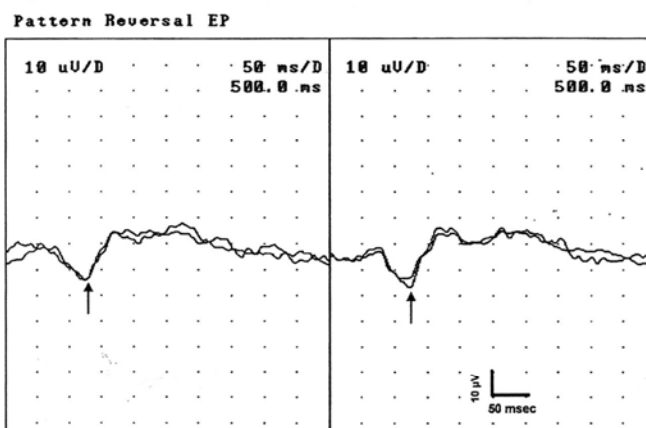


Fig. 3 Visual potentials (VEP) to reversal pattern in patient #5: note a bilateral increased latency of P100 wave (125 msec and 117 msec, respectively; maximum normal values 109 msec and 107 msec, respectively).

binemia in childhood. This hypothesis is also supported by the observation that EEG abnormalities were present only before transplantation in the patient examined before and after this treatment. EEG was abnormal in all overlap type I/II CN subjects who had been chronically exposed to both high levels of serum bilirubin and a long period of exposure (mean age at neurophysiological evaluation: 20.7 years); while the EEG was normal in classic type II CN. These findings indicate there is a tight relationship between EEG abnormalities and serum bilirubin levels, as previously reported [15,19], confirming cortical bilirubin neurotoxicity.

According to the same studies [15,19], brainstem auditory pathways were undamaged in all ten patients who underwent BAEP studies, suggesting a poor vulnerability of brainstem auditory pathways to the effect of bilirubin in respect to other central nervous system areas [21].

Central motor conduction delay obtained by TMS was the most frequent neurophysiological finding in our patients (five out of nine, including two out of four transplanted subjects), notwithstanding these patients did not show evident clinical signs of pyramidal impairment. In our series, unlike VEP and EEG findings, MEP alterations were not correlated to CN type and bilirubin levels. In fact, a prolonged central motor conduction time was observed in two out of three patients with lower bilirubin values (classical type II CN; BR range: 4–9 mg/dL), thus MEPs are a useful marker for motor cortico-spinal pathway damage but not for the management of CN patients.

In contrast with previous studies that reported normal VEPs in CN I syndrome [15,19], we found visual pathway abnormalities at VEP in half of our patients with type I and overlap type I/II CN syndrome. In type I CN these abnormalities were found several years after liver transplantation.

VEPs follow-up performed in five patients showed that they were persistently normal in one transplanted CN I, one overlap type I/II CN, and one type II patient. In two overlap type I/II CN patients the follow-up revealed an electrophysiological worsening and a transition from normal to pathological pattern, respectively.

VEPs abnormalities were still present in transplanted patients even in the absence of visual overt symptoms and EEG abnormalities. This suggests that they are the most sensitive electro-

physiological test in regards to a previous long exposure to neurotoxic hyperbilirubinemia and that it is not possible to rely only on EEG results to rule out sequelae in these patients.

Available therapies of severe hyperbilirubinemia of CN syndrome are far from being optimal and some of them may lose effect over time (e.g., phototherapy: increasing surface area and skin thickness; poor compliance to its intensive regimen with a great impact on the life quality). Tin-protoporphyrin is burdened by several side effects (e.g., skin photosensitivity). The hepatocyte or stem cell transplantation procedure is still experimental but has shown promising results [10] and it may be applied to bridge patients to OLT [1]. Gene therapy is not practically available and for safe and definite treatment there is still a long way to go [16]. Liver transplantation has been demonstrated to be effective [9] and no specific neurotoxic effect of calcineurin inhibitors has been described even in those CN patients who had been transplanted having overt or subclinical neurological impairment. Its risk versus benefit ratio, however, is undetermined because of possible significant transplant-related complications. One major point is the best age to transplant CN patients and appropriate timing of the procedure before irreversible neurological damage occurs which also remains an unresolved issue [20,24]. In fact, the literature shows the possibility of a sudden severe clinical neurological damage consisting in cognitive decline, pyramidal, extrapyramidal and cerebellar dysfunction in untreated type I CN patients [12,18,22], and death or lesions due to kernicterus in severe type II CN patients [3,4,7,8].

All four CN I patients had unremitting very high serum bilirubin values and bilirubin/albumin molar ratios which required continuous intensive phototherapy from the first months of life. Since it became less effective with age, compliance to a treatment with more hours/day was obviously not compatible with a social life. Their families agreed therefore for a transplant at a rather young age (mean 6.8 years), that is in the usual range described in the literature [20,24].

Conversely, none of the patients with CN I/II clinical phenotype has hitherto been transplanted mainly because of their own or their families long-lasting hesitation to the invasiveness of this surgical therapeutic option without major subjective worsening of clinical status. Initial response to PB in fact was sufficient to

keep bilirubin values under adequate control. Later on, however, they needed to add phototherapy and in cases of intercurrent illness, other medical treatments (e.g., plasmapheresis followed by albumin infusion). In particular, patient # 5 who has bilirubin/albumin molar ratios often near to a critical value of 0.7 [20], is felt by the medical team to be at high risk for sudden irreversible neurological deterioration and is at present on the active waiting list for liver transplantation. He has undergone more frequent episodes of hyperbilirubinemia poorly responsive to therapies, that were accompanied by a significant worsening of P100 latency. As previously stated, the long-term treatment with nocturnal phototherapy in these patients becomes less effective with age and is highly disruptive to their social life. These factors will probably play a role in the decision of undergoing liver transplantation as the definitive treatment also for the other two patients with CN I-II.

Our findings, although numerically limited in this pilot study, show that neurophysiological tests, in particular VEPs, may be useful for clinical management of CN syndrome to identify and monitor neurotoxic effects of marked hyperbilirubinemia. This may be particularly useful in the subgroup of CN type II cases who have very severe hyperbilirubinemia poorly responsive to PB in the absence of overt clinical neurologic damage. In fact some hesitation for liver transplantation exists on the part of these patients and/or their families who hope gene therapy might become available soon. Of course, it is important to note that the neurological tests in the state of compensation can only provide limited guidance as to the timing of transplantation because of the risk of sudden deterioration following an acute intercurrent illness.

In conclusion, both EEG and VEP abnormalities seem to be related to serum bilirubin levels. However, since VEPs are a quantitative test compared to EEG evaluation, we propose VEP testing as a useful ancillary tool to monitor the neurotoxic effects of hyperbilirubinemia. In stable patients VEPs may be tested at longer intervals (e.g., yearly). Stricter monitoring should be performed in patients who became unresponsive to medical therapies when BR levels or Bil/Alb molar ratios stay in a critical range which could worsen along with unpredictable acute BR increases. Although the need for OLT is normally driven by the severity of the clinical and biochemical background, in this selected group of patients the finding of EEG and VEP abnormalities may be helpful in the decisional process regarding the necessity of a rapid pre-transplant evaluation before neurological deterioration happens. Bilirubin and bilirubin/albumin ratios should be assessed at the time of neurophysiological monitoring.

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3.1. POST TRANSPLANT ERYTHROCYTOSIS AFFECTING FIVE LIVER RECIPIENTS

INTRODUCTION

Erythrocytosis is a quite common complication of renal transplant recipients with a prevalence on the order of 10-15% (1) varying from as low as 3.2% in the pediatric age (2) to as high as 20.2% in adults (1,3), and has not been reported in other types of organ transplantation. A transient increase in endogenous erythropoietin levels (EPO) after 12-48 hours from orthotopic liver transplantation (OLT) has been described in six out of ten liver transplanted children (4). Its recognition and treatment are important for maintaining hematocrit (Hct) below the critical threshold of 51% to reduce the clinical symptoms and/or to minimize the possible risk of hyperviscosity related thrombosis especially at sites of vascular anastomosis (1,5-7). The pathogenesis is multifactorial and still poorly understood. EPO, the renin-angiotensin system, male gender and renal cysts are some of the factors that have been proposed to play a role in post-renal transplant erythrocytosis (1,3,7-10).

AIM

To investigate wheter and why erythrocytosis may develop also in the long term course after orthotropic liver transplantation (OLT).

PATIENTS AND METHODS

We retrospectively evaluated the occurrence of erythrocytosis in ninety Italian liver transplanted children (F:M=42:48, age: 12.8 ± 6.7 years) followed up at our department. Mean age (\pm SD) at liver transplantation was 3.2 ± 3.6 years (range 0.2 – 12.5). Main

OLT indication was Biliary atresia (70%). Primary immunosuppressor was Tacrolimus in 64 (71.2%) and Cyclosporin A (CsA) in 26 (28.8%) patients. Family and personal history, complete physical examination, symptoms possibly related to erythrocytosis (e.g. headache, malaise, dizziness), red blood cells (RBC) count, hemoglobin levels (Hb), Hct, calcineurin inhibitors levels, hepatic and renal function tests including ultrasonographic (US) parameters were recorded from their files.

Erythrocytosis was suspected based on increased Hb and Hct values ($>$ mean +2SD or $>$ 97th percentile) in two separate blood counts (11). Age-specific reference ranges have been considered (12). Patients with erythrocytosis underwent diagnostic tests and procedures aiming to identify possible causes of an absolute erythrocytosis.

Therefore EPO receptor mutation and JAK2 V617F mutation, the last recently reported in the majority of adult patients with Polycythemia Vera (13), were searched. Exon 12 of JAK2 was also analyzed to investigate the possible mutations described in patients with Polycythemia Vera who did not have V617F mutation (14) Renin serum levels, high oxygen-affinity haemoglobins (defect of α and β globin chains) and gene mutations of the oxygen sensing pathway (VHL, PHD2, HIF-2 α) were evaluated as well. Central hypoxia driven processes due to smoke, high altitude, chronic lung disease, sleep apnea and congenital cyanotic

heart disease were investigated by personal-history, arterial blood gas analysis, chest x-ray, spirometry, otorhinolaryngologic evaluation and echocardiogram. A total-body computed tomography (CT) was obtained to exclude malignant and non-malignant tumours associated to a pathological EPO production only in patients with increased EPO levels.

RESULTS

None of 90 children had elevated pre-OLT Hct values, and no renal cysts were found at routine abdominal US and CT performed before OLT. As shown in table 1, four male and one female patients (5.5%) showed erythrocytosis at 14.8 ± 4.3 years of age (RBC count, Hb and Hct above normal values). The mean time (\pm SD) from transplantation to diagnosis of hematological disorder was 9.1 ± 5.1 years. Extended family history was negative for kidney cystic diseases. All brothers and sisters, and both parents of the five patients had normal complete blood counts and renal ultrasonographic images. None of the patients were smokers or lived at high altitude. None of them was affected by a congenital cyanotic heart disease as demonstrated by routine echocardiograms performed before OLT. Physical examination of the patients did not show relevant abnormalities. Platelets and white blood cell counts, RBC morphology, mean corpuscular volume, and spleen size were within normal ranges except for two patients (CD, AL) who had splenomegaly and a progressive decrease of platelet count. We did not find V617F and exon 12 mutations of JAK2. EPO receptor abnormalities were absent in all patients. Renin serum levels, Hb electrophoresis, mutations of oxygen sensing pathway genes (VHL, PHD2, HIF-2 α), repeated arterial blood gas analysis, chest x-ray, spirometry were negative or within normal limits. The profile of biochemical tests to survey the function

of other principal body organs and systems including liver and kidney was normal (table 1; figure 1).

Three of the five patients (NG, RC, CD) were asymptomatic and developed erythrocytosis 8.5, 4.1 and 14.1 years post-OLT, respectively. Their EPO levels were within normal limits (figure 1). Kidney ultrasonography and CT were negative for renal cysts. Primary immunosuppressor was Tacrolimus in the first two, whole blood levels being within normal limits (range 4-6 ng/ml) (table 1). In the third patient (CD) primary immunosuppression consisted initially in Csa and after 9 years from OLT it was substituted by Tacrolimus because of early signs of chronic rejection. During follow up, no symptoms related to erythrocytosis were reported in all and Hct values fluctuated ranging between 48.3% and 52.7% with the exception of the last case (CD) who showed increased Hct values ($>50\%$) in three separate blood counts in presence of Hb values persistently increased (mean \pm SD: Hb 16.9 ± 0.6 g/dl). The only one female patient (AL), who was taking Tacrolimus, developed erythrocytosis earlier than others (i.e. 4.4 years after OLT). Her Hb (mean Hb \pm SD: 15.9 ± 0.4 g/dl) and Hct values (mean Hct \pm SD: 45.5 ± 1.1 %) were increased referring to age-specific reference ranges (12) such as EPO levels that were fluctuating and sometimes elevated ranging from to 13-25 mU/ml. However there was no evidence of renal cysts at US investigation (table 1; figure 1). In the last six months a transient isolated and asymptomatic hypoxia was occasionally discovered by arterial blood analysis. Oxygen saturation level (SaO_2), inflammatory and infectious parameters, chest x-ray, spirometry and echocardiogram were negative. As shown in Table 1, the fourth patient (RT) developed late clinically symptomatic erythrocytosis at age fifteen (14.5 years post-OLT). EPO serum levels were twofold the upper normal limits. Two years

before, multiple cysts with a diameter ranging from 0.3 to 0.6 mm in both kidneys appeared at ultrasonography. A CT total-body resulted negative for aberrant EPO producing mass and confirmed renal cysts. Primary immunosuppressor was CsA, and its whole blood levels were within therapeutic range for post-transplant age (ranging 70-100 ng/ml, monoclonal fraction). Two months after erythrocytosis onset he had to switch to Tacrolimus because of mild signs of rejection observed at a routinely planned liver biopsy in the presence of normal liver function tests.

During a five-year follow up, several phlebotomy (n=10) and erythroapheresis (n=14) sessions were necessary to maintain Hct in a safe range and to reduce clinical symptoms. An ACE inhibitor (Ramipril) was furthermore added to the treatment because of persistence of clinical and laboratory abnormalities. Response was initially poor. At age 20, MRI showed enlarged cysts with major diameter of 16 mm. In the last year EPO levels reached approximately four folds the upper normal limits. However Hct and Hb levels become normal after 18 months so that phlebotomy or erythroapheresis procedures were not necessary. Present EPO level is still high (31.3 mU/ml)

DISCUSSION

Post- transplant erythrocytosis is a complication that has hitherto been described only in renal graft recipients, mainly adults (1-3,7). A transient erythrocytosis condition has been reported only in one series of children soon after liver transplantation (OLT) (4). Here we describe five cases of erythrocytosis developing during the long term follow-up after OLT.

Differently from renal transplanted patients, where erythrocytosis develops during the first years of follow-up (1), our patients presented this condition later (4.1-14.5 years after OLT).

The pathogenesis of post-transplant erythrocytosis remains still incompletely understood and it is believed to be multifactorial (1,3, 7-10). In keeping with post-renal transplant erythrocytosis reports (1,7,9) our symptomatic patient showed high EPO levels, contrary to expectation from the normally negative feedback loop between Hct and EPO secretion (1). Because renal function was normal and no subclinical hypoxia was detected by repeated arterial blood gas analysis, we believe that excess EPO production might be related to the presence of the renal cysts. It is unclear whether renal cysts produce EPO or simply cause local ischemic injury by compressing adjacent renal tissue leading to local renal hypoxia and to increased EPO production (7,11). The possible association between renal cysts and erythrocytosis is supported by the resolution of this condition after drainage or resection of cysts in some case reports of secondary polycythaemia which as been described in non-transplanted patients (15,16). In a large series of 108 liver transplanted children an high incidence (30%) of post-OLT acquired renal cystic disease was detected by CT scan (17). A lower incidence (11%), comparable to our results (8.8%), was observed with ultrasonography (US) in another recent series of 235 OLT-children (18). The different incidence of renal cysts is probably due to the less sensitivity of US vs. CT scan (17). Unexpectedly, no mention of erythrocytosis was made in these studies.

In our remaining clinically asymptomatic patients who had normal EPO levels and no renal laboratory and ultrasonographic alterations, the mechanisms underlying erythrocytosis remains unknown. In these male patients renin-

angiotensin system and endogenous androgens might play a role. These hormones may stimulate directly the erythroid progenitor lines or may augment the production of other erythropoietic factors (1). Abnormal erythroid precursor sensitivity to EPO might also be implicated (6,10). It is interesting to note the case of the young female patient who had increased and fluctuating EPO levels with a transient asymptomatic hypoxia occasionally discovered by arterial blood gas analysis. However we do not know if a correlation between these laboratory data and the increased EPO serum levels exists. In all cases, most causes of erythrocytosis were ruled out by a number of tests performed during follow-up.

In the majority of renal transplant studies erythrocytosis was more common in male patients (1,3,15) and in those who received CsA (1,3). In our series erythrocytosis developed in four male patients: two of them were receiving Tacrolimus, while the other two patients at first were receiving CsA. This may suggest that in OLT recipients Hct abnormalities may develop and go on independently of the type of immunosuppressive therapy. The possible responsibility of immunosuppressive agents remains uncertain also because the speculated drug related effect developed several years after OLT.

In adults, erythrocytosis treatment is considered necessary to reduce the potential thromboembolic risk secondary to blood hyperviscosity (1,5-7). Phlebotomy/erythroapheresis is the first line standard treatment (18). Some drugs, such as angiotensin converting enzyme inhibitors, angiotensin-II receptor antagonist and adenosine receptor antagonist have been reported to reduce Hct elevated values in kidney recipients (1,19). In particular it has been demonstrated that Ramipril may be effective in the post-renal transplant erythrocytosis; low doses normalized Hct in most patients (19). In our symptomatic patient phlebotomy/erythroapheresis were well tolerated and no

severe iron deficiency developed. Because of persistence of clinical and laboratory abnormalities, Ramipril was introduced. Although its response was initially modest, Hct and Hb levels become normal after 18 months of therapy. In the other patients, due to absence of clinical symptoms and to fluctuations of Hct values, no therapy has still been started. Pending more definite management criteria (6), a longer follow up was thought to be necessary to evaluate the course of the erythrocytosis and the need for treatment.

In conclusion, we have shown that erythrocytosis may be a rare and late complication in patients who underwent liver transplantation in the pediatric-adolescent age. The pathogenesis remains still incompletely understood. EPO, male gender, and renal cysts may probably play a role. Future research may still investigate on JAK2 exon12 mutations, which have most recently been described both in polycythemia vera and idiopathic erythrocytosis (14).

Epidemiological studies on erythrocytosis in children overall, and in post OLT patients in particular, are necessary to establish whether a casual (as seen in post renal transplantation) rather than a causal association may exist between this hematological abnormality and liver transplantation.

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TABLE 1 Demographic, clinical and laboratory findings of five patients who developed post-liver transplantation erythrocytosis (PTE)

| Patient | NG | RC | CD | AL | RT |
|---|---------------------|--------------------|--------------------|---|--|
| Gender | M | M | M | F | M |
| OLT indication | Crigler Najjar I | Biliary Atresia | Biliary Atresia | Biliary Atresia | Biliary Atresia |
| OLT Age (yr) | 10.3 | 12.5 | 1.9 | 3.1 | 0.8 |
| Age at PTE diagnosis (yr) | 18 | 16.6 | 16 | 7.5 | 15.3 |
| Immunosuppressor at PTE diagnosis | Tacrolimus | Tacrolimus | Tacrolimus | Tacrolimus | Cyclosporin A |
| Hct pre-OLT (%) | 37.2 | 37.1 | 31.1 | 35 | 32.8 |
| Hct at PTE diagnosis (%) | 51.5 | 51.7 | 47.7 | 47.8 | 53.1 |
| EPO at PTE diagnosis (mU/ml) (n.v. 3-20) | 14 | 13 | 13.8 | 17.5 | 38 |
| Hb pre-OLT (g/dl) | 12 | 12.6 | 10.2 | 12 | 10.5 |
| Hb at PTE diagnosis (g/dl) | 17.6 | 17.7 | 17.3 | 16.3 | 18.0 |
| RBC pre-OLT (x 10⁶/μL) | 5.12 | 5.20 | 3.66 | 4.14 | 3.12 |
| RBC at PTE diagnosis (x 10⁶/μL) | 6.14 | 6.17 | 5.20 | 5.45 | 5.68 |
| Creatinine clearance at PTE diagnosis (ml/min) | 119 | 116 | 101.7 | 72.6 | 91 |
| Renal US/TC at PTE diagnosis | Normal | Normal | Normal | Normal | Multiple cysts in both kidneys |
| Signs and symptoms related to PTE | No | No | No | No/transient asymptomatic hypoxia | Yes (headache, malaise, dizziness, hypertension) |

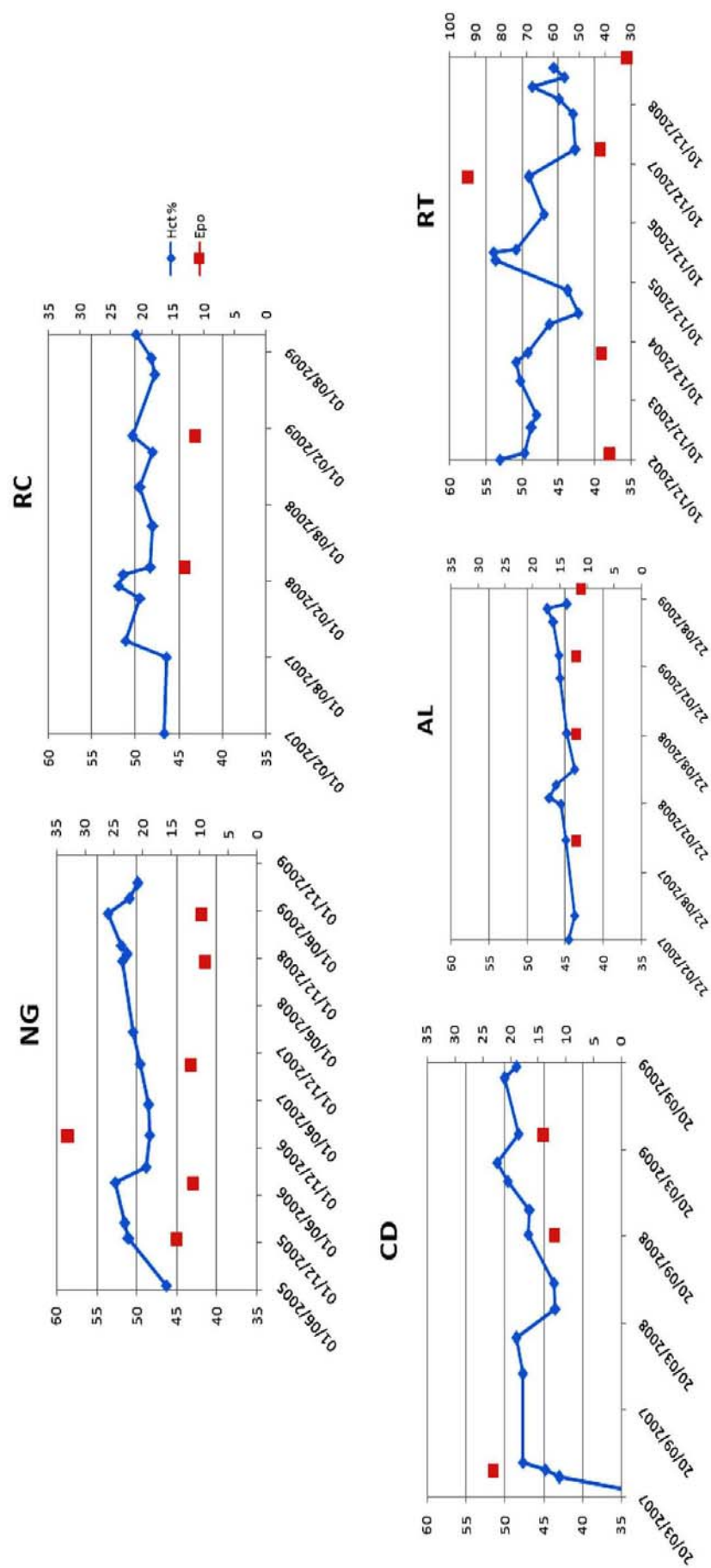


FIGURE 1. Hct and EPO time course values of 5 patients who developed post-liver transplantation erythrocytosis

POST TRANSPLANT ERYTHROCYTOSIS MAY AFFECT ALSO LIVER RECIPIENTS

M. Caropreso, M. Esposito, G. Capuano, S. Maddaluno, S. Lenta, N. Di Cosmo, I. De Napoli, C. Veropalumbo, G. Crispino, R. Iorio, P. Vajro

Department of Pediatrics, University of Naples Federico II, Naples, Italy

Aims. Post transplant erythrocytosis is a quite common (10–20%) complication which has been described only in kidney recipients (Demetrios et al., *Kidney Int*, 2003). Its pathogenesis remains unknown: erythropoietin (EPO), the renin-angiotensin system, male gender, and presence of renal cysts are some of the factors that have been proposed to play a pathogenetic role. Here we describe three cases of erythrocytosis after liver transplantation.

Patients and methods. We retrospectively evaluated ninety liver transplanted children (F:M=42:48; age: 12.8 ± 6.7 years) followed-up at our institution. Mean age at liver transplantation (OLT) was 3.55 ± 3.65 years (range 0.2–12.5 years). Main OLT indication was Biliary Atresia (70%). Primary immunosuppressor was Cyclosporine in 26 and Tacrolimus in 64 patients. Complete physical examination, symptoms possibly related to erythrocytosis, red blood cell (RBC) count, hemoglobin levels, hematocrit (Hct), renal and hepatic function tests including ultrasonographic parameters were recorded. Hemoglobin electrophoresis, hemogasanalysis, spirometry and EPO were investigated in those with erythrocytosis [persistent Hct above 51%].

Results. None had abnormal Hct before OLT. Three male liver recipients (3.3%) developed erythrocytosis (first abnormal Hct values: 51.2, 51.5 and 53.1%) at a mean age of 16.9 ± 1.77 years. The mean time from transplantation was 9.03 ± 5.22 years. All three patients received primary immunosuppression with Tacrolimus. The main potential causes of erythrocytosis were ruled out since personal history for tobacco smoking, hemoglobin electrophoresis, hemogasanalysis and spirometry were negative or within normal limits. Red blood cell (RBC) count was above normal values (range $5.8\text{--}6.2 \times 10^6/\mu\text{l}$). RBC morphology, mean corpuscular volume, platelets and white blood cells count, liver and kidney function tests were within normal range. Kidney ultrasonography showed multiple anechogenic cysts in both kidneys in one patient (RT).

EPO levels were normal in two (14 and 13 mU/ml, n.v.: 3–20 mU/ml) and high in one (pt RT) (Epo: 38–90 mU/ml). The latter patient was also the only individual with symptoms related to erythrocytosis: headache, malaise and dizziness. During a five year follow-up he needed several phlebotomy or erythroapheresis settings, with a variable frequency (4–9 setting per year) to maintain Hct < 51% and reduce the risk of thromboembolic events. During the last 2 years ACE inhibition (Ramipril) was added to the treatment, but it was poorly efficacious.

Conclusion. Our three patients represent the first paediatric report of erythrocytosis after liver transplantation.

The pathogenesis of post-transplantation erythrocytosis is still incompletely understood.

In our symptomatic patient excess EPO production may probably be related to the presence of the renal cysts. It is unclear whether these produce EPO or simply cause local ischemic injury by compressing adjacent renal tissue leading to increased EPO production.

More studies are necessary to establish the incidence and the pathogenetic mechanisms of erythrocytosis in liver transplanted children. This information may be useful to plan early treatment to reduce the thromboembolic risk.

ERYTHROCYTOSIS: ALSO A POSTTRANSPLANT COMPLICATION IN LIVER RECIPIENTS?

Presenter: P. Vajro. *Università di Napoli, Napoli, Italy.*

Co-authors: S. Maddaluno¹, M. Caropreso¹, C. Veropalumbo¹, M. Esposito¹, I. De Napoli¹, A. Varriale¹, C. Gentile¹, R. Iorio¹. ¹*Dipartimento di Pediatria Università di Napoli "Federico II," Napoli, Italy.*

Background and Aim: Erythrocytosis may complicate renal transplantation with a prevalence of the order of

10–15% varying from as low as 3.2% in the pediatric age to as high as 20% in adults and has not been reported in other types of organ transplantation. It is suggested that factors like erythropoietin (EPO), renin-angiotensin system, male gender, and renal cysts may play a role. We report 3 cases of erythrocytosis developing after liver transplantation (OLT).

Methods: 90 Italian OLT children (F=42; age: 12.8 ± 6.7 years) followed up at our institution were retrospectively evaluated. Mean age at OLT was 3.5 ± 3.6 years (range 0.2 – 12.5 years); biliary atresia was the main OLT indication (70%). Cyclosporin A and tacrolimus were the primary immunosuppressors in 26 and in 64 patients, respectively. Family and clinical history, complete physical examination, blood cells count, hemoglobin levels, hematocrit (Hct), renal and hepatic function tests including ultrasonographic parameters were recorded. Hemoglobin electrophoresis, arterial blood gas analysis, spirometry, EPO, and abdominal CT scan or NMR were investigated in patients with Hct persistently > 51% to rule out main causes of erythrocytosis.

Results: Hct was normal in all patients before OLT. Three male liver recipients receiving tacrolimus as primary immunosuppressor developed persistent erythrocytosis at ages 18.8, 16.6, and 15.3 years. Their Hct values at the time of diagnosis were 51.2%, 51.5%, and 53.1%, respectively. The mean time from transplantation was 9.0 ± 5.2 years. Red blood cells (RBC) showed values ranging from 5.8 to $6.2 \times 10^6/\mu\text{L}$. RBC mean corpuscular volume and morphology, platelets and white blood cells count, liver and kidney function tests were in the normal range. Clinical, laboratory, and instrumental data excluded the main potential causes of erythrocytosis in all but 1 patient who had erythrocytosis related symptoms (headache, malaise, and dizziness), multiple bilateral simple renal cysts and high EPO levels (38–90 mU/mL, n.v. 3–20 mU/mL). During a 5-year follow-up he needed several erythroapheresis settings and ACE inhibition.

Conclusions: These are the first 3 cases of erythrocytosis developing after OLT. In the symptomatic patient erythrocytosis may be due to high levels of EPO: it is unclear whether renal cysts may increase EPO production directly or by local ischemia of adjacent renal tissue. Our preliminary results suggest the need of searching for erythrocytosis also in OLT recipients' follow-up.

3.2 SELF CONCEPT AND PSYCHOPATOLOGICAL RISK IN LIVER TRANSPLANTATED CHILDREN

INTRODUCTION

In the last 20 years, liver transplantation has become the treatment of choice for several pediatric liver diseases that progress to end-stage liver failure (1). Graft and patient survival continue to improve due to developments of medical and surgical management, new immunosuppressant drugs, higher organ availability and control of post-operative complications (2). Currently patients' long-term survival rates are reported to be between 80 and 90% (1-4). The possibility of prolonging recipient's life expectancy triggered several studies which focus not only on organic problems but also on neurological and psychological aspects of childhood orthotopic liver-transplantation (OLT) (5).

It has been reported that children who have undergone OLT may present emotional and behavioural disturbances for a long period of time after transplantation and it was higher than in children with chronic liver diseases (5,6). Due to small size of studied series, several uncertainties regarding psychological problems in these patients and the need to differentiate their disturbances from those related to the chronic liver disease itself however still exist. Evaluation of self-concept, an area poorly explored in OLT recipients (7), needs to be studied as well.

AIM

To assess psychopathological risk and self-concept in liver transplanted children as compared to age matched controls with stable chronic liver disease.

PATIENTS AND METHODS

Patients

29 OLT patients with a mean age of 11.7 ± 3.9 years (range 6–18) have been enrolled for this study. Patients have been compared with an age matched control group (CTRL) affected by stable chronic liver disease. All children were regularly attending school. No patient or family was or had been involved in longterm or intensive psychosocial support programs during any phase of the transplantation process.

Methods

The assessment included individual sessions and testing procedures as follows:

1. CBCL (Child Behaviour Checklist) for assessing behaviour problems and competences of children aged 4–18 years (8,9). It consists of 113 items, completed by parents, listing a range of behavioural and emotional symptoms, rated using a semiquantitative scale. The author of this test developed a model consisting of eight subscales or Syndromic Scales: Withdrawal, Somatic Complaints, Anxiety/Depression, Social Problems, Thought Problems, Attention Problems, Delinquent- Aggressive Behaviour, as well as a scale for Sex Problems. The test, includes, moreover, three additional scores: one for Internalization, one for Externalization and one for Total

Problems. Competences are assessed by other three scales: Activities, Social Function and School. A total score is obtained by summing up these scales. CBCL profiles enable to discriminate Normal, Borderline and Clinical children (8,9).

2. TMA (Test Multidimensionale dell'Autostima) is the Italian version of the MSCS (Multidimensional Self Concept Scale) (10). It consists of 150 Likert-type items and was designed for either individual or group administration. The TMA reflects a context-dependent, multidimensional self-concept model. TMA assesses self-concept in each of the following six subdomains: Social, Competence, Affect, Academic, Family and Physical. The TMA manual reports .98 total scale internal consistency and .90 stability (i.e., 2 weeks). TMA subscale internal consistency ranges from .87 to .97, and subscale stability coefficients range from .73 to .81. The validity of TMA and its multidimensional, context-dependent model have been studied among runaway adolescents, with respect to students' age, race, and gender, as the model and test relate to children sociometrically determined social status (10).

Statistical analysis

Difference between the number of patients with normal results obtained by CBCL and TMA scores, and patients with borderline and pathological results has been calculated by exact Fisher test (EFT). A significant value has been identified by $p \text{ value} < 0.05$.

RESULTS

Patients' mean age at the time of OLT was 3.2 ± 3.5 years (range 0.7–9.8). The mean time elapsed since transplantation was 5.1 ± 4.5 years (range 0.7–15.8). Biliary Atresia was the main indications to OLT (90%).

CBCL test and TMA results are shown as follow:

1. CBCL test results.

Total Problems Scale showed a statistically significant higher percentage of results within the pathological range in the OLT group (52%) vs CTRL group (17%) ($p = 0.03$). Total Competence Scale showed results within the pathological range in both groups (88% and 92% in OLT and CTRL groups, respectively) with no statistically significant difference ($p = 0.67$). The increased number of pathological results in the Total Competence Scale was preminently due to the Activity section.

2. TMA results.

With regard to Self-Concept there were no statistically significant differences between the two groups concerning Social, Competence, Affect, Family and Physical subdomains. In the Academic subdomain there was a statistically significant difference in the percentage of patients showing a lower self-concept (50% and 9% in OLT and CTRL groups, respectively) ($p = 0.025$).

DISCUSSION

A mild cognitive deficit and moderate neuropsychological dysfunctions due to organic brain damage have been reported in the early studies related to mental development of pediatric OLT recipients (11-13). Length of previous

hospitalizations, young age at onset of liver disease and poor nutritional status have been postulated to play a role in these deficits. It remains controversial as to whether developmental delay improves significantly after OLT (6). The constant need for medical supervision and daily use of immunosuppressive medication put these patients at risk of developing psychological problems similar to other patients with chronic illness (14). In general there is consistent agreement that children who have undergone OLT, may present emotional and behavioural disturbances (15,16). Some studies demonstrate the presence of psychological difficulties such as anxiety/depression, aggressiveness, mild cognitive deficits and school problems (15,17,18). There is still poor information on child's psychological profile and psychosocial effects on family life well being following OLT (19-21). Our group has already given a preliminary contribute to evaluation of psychological impact on children who underwent liver transplantation. We suggested that the personality of OLT recipients was the result of two stressors: the early onset of chronic disease and the transplantation itself. These data also indicated that emotional and behavioural disturbances were often present long term after OLT (6). Subsequently, by using quantitative assessment methods, we confirmed in a small number of patients the hypothesis that children who have undergone OLT may present a psychopathological risk as compared with an age and gender matched control group affected by stable chronic liver disease (5). This risk, furthermore, seemed to exist for a long period of time after transplantation (5). In the present study we have confirmed in a larger series of OLT children our previous data. In fact CBCL evaluation has shown that they have an higher risk for behavioural and emotional disorders as compared to general and hepatopatic peers, and that both groups have reduced competences due to pathological Activity Scale results.

Difficulties observed in the social adjustment need further investigation by evaluating also quality of life (14). Concerning TMA, low scores found in the Academic scale underline the existence of lower school activity performances in OLT patients who may therefore require a special support.

In conclusion, our results are in tune with the concept that the success of liver transplantation should not be measured only by survival rates. Its outcome will need to be judged using a measure which incorporates both survival rates and several features of children life including psychological and social well being as well.

Understanding of the interaction between OLT and patient's psychopathological risk and long-term health needs after liver transplantation is necessary to offer anticipatory guidance and targeted interventions.

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SELF-CONCEPT AND PSYCHOPATOLOGICAL RISK IN LIVER TRANSPLANTED CHILDREN

M. Caropreso^a, I. De Napoli^a, T. Gasparro^b, S. Pisano^b, S. Maddaluno^a, M. Esposito^a, L. Pezzuti^b, C. Veropalumbo^a, A. Gritti^b, R. Iorio^a, P. Vajro^a

^a *Department of Pediatrics, University of Naples Federico II, Naples, Italy*

^b *Chair of Child Neuropsychiatry, Second University of Naples (SUN), Naples, Italy*

Aims. Increased recipients' life expectancy in children undergoing orthotopic liver transplantation (OLT) has triggered several studies focussing not only on organic problems but also on neurological and psychological aspects.

Aim of the present study is to assess psychopathological risk and self-concept in liver transplanted children as

compared to age matched controls with stable chronic liver disease.

Material and methods. 29 OLT patients with a mean age of 11.7 ± 3.9 years (range 6–18) have been enrolled for this study. Mean age at the time of OLT was 3.2 ± 3.5 years (range 0.7–9.8). The mean time elapsed since transplantation was 5.1 ± 4.5 years (range 0.7–15.8). The main indications to OLT was Biliary Atresia (90%). Patients have been compared with an age matched control group (CTRL) affected by stable chronic liver disease. All children were regularly attending school. No patient or family was or had been involved in long-term or intensive psychosocial support programs during any phase of the transplantation process.

The assessment included individual sessions and testing procedures as follows:

1. CBCL (Child Behaviour Checklist) for assessing behaviour problems and competences of children aged 4–18 years;
2. TMA (Test Multidimensionale dell'Autostima) which is the Italian version of the MSCS (Multidimensional Self-Concept Scale). The TMA assesses self-concept in each of the following six subdomains: Social, Competence, Affect, Academic, Family and Physical.

Results. 1. CBCL test. Total Problems Scale showed a statistically significant higher percentage of results within the pathological range in the OLT group (52%) vs CTRL group (17%) ($p=0.03$ at exact Fisher Test, EFT).

Total Competence Scale showed results within the pathological range in both groups (88% and 92% in OLT and CTRL groups, respectively) with no statistically significant difference ($p=0.67$ EFT).

2. TMA. With regard to Self-Concept there were no statistically significant differences between the two groups concerning Social, Competence, Affect, Family and Physical subdomains. Only in the Academic subdomain there was a statistically significant difference in the percentage of patients showing a lower self-concept (50% and 9% in OLT and CTRL groups, respectively) ($p=0.025$, EFT).

Conclusion. CBCL results confirm that OLT patients have an higher risk for behavioural and emotional disorders as compared to general and hepatopatic peers. OLT and hepatopatic patients difficulties in the social adjustment need further investigation by evaluating also quality of life. Concerning TMA, low scores which have been found in the academic scale underline the existence of activity school lower performances in OLT patients who may therefore require a special support.

CONCLUSIONS

WHAT THESE STUDIES ADD TO THE CURRENT KNOWLEDGE AND WHAT ARE THE RECOMMENDATIONS FOR FUTURE RESEARCH

A key purpose of this research is to contribute to a better management of children affected by liver disease by exploring several pathogenetic, diagnostic and natural history aspects. Our project is thus a series of essays, each of which evaluates and synthesizes data to generate a knowledge of several unclear aspects that might be applied to clinical practice. Therefore, each chapter is intended to highlight particular and emerging facets of liver disease to improve available tools for the care of hepatopathic patients. Most of our data are not conclusive but we believe that may in part be already transferred to patients' management and also be useful to trigger further research in this area as well.

1. Regarding the study of genetic prothrombotic risk factors in children with extrahepatic portal vein obstruction, we have shown that a congenital condition of thrombophilia is found in one third of these patients, suggesting that prothrombotic disorders may be involved in the development of childhood PVT. Pediatricians ensuring the long-term follow-up of infants needing neonatal intensive care should consider the possibility of PVT in their patients. In our series it is interesting to note that pre-existing stress of portal vein by catheterism seems to facilitate development of PVT especially in genetically predisposed individuals. Differently from adults V617F JAK2 probably does not play a role in childhood PVT. Understanding of thrombophilic genetic factors predisposing to PVT could be decisive to identify at risk patients subgroups and prevent other

possible thrombotic events also in different districts. This may lead to approach these patients appropriately so that their quality of life can be improved.

Since patients' number of previous studies deals with small series and no definite conclusions may be drawn, to gain a better insight into pathogenesis future research should be performed on large scale projects. Further evaluation of the V617F and possibly exon 12 JAK2 are recommended to assess their potential pathogenetic role and to recognize patients who probably should be carefully observed for the subsequent overt MPD.

2. As for prevalence and long-term course of macro-aspartate aminotransferase (macro-AST) in children, our study is the first that has hitherto evaluated these issues. Given the large number of enrolled children, we were able to calculate with reasonable accuracy the prevalence of macro-AST (more than one third cases) among children with isolated abnormal serum AST levels. The lack of pathological correlates over a long period argues for the benign nature of this phenomenon in childhood. We identified polyethylene glycol (PEG) precipitable activity (%PPA) thresholds vs. electrophoresis, the gold standard diagnostic test. Our % PPA thresholds will allow to use this technique as a simple and precise screening test, electrophoresis being reserved for confirming positive screen tests and cases in which %PPA levels are of intermediate discriminant accuracy. Physicians should be aware of the asymptomatic macro-AST phenomenon in the absence of a disease state and also to consider this diagnostic possibility at an early stage when studying patients with isolated, raised AST. Prior documentation of macro-AST condition in a patient should be given the same prominence in a patient's medical record as drug allergies. This way, invasive, costly, or even, potentially life-threatening investigations (e.g. needle liver biopsy) or treatment may be averted. Moreover,

it is important to reassure affected children and their families that –according to our results- macro-AST has a good evolution and does not require any specific treatment.

Future research on larger series of patients should evaluate the applicability and validity of our % PPA thresholds.

3. Our prospective study on clinical utility of electrophysiological evaluation in Crigler-Najjar syndrome has shown that only electroencephalogram (EEG) and visual evoked potentials (VEPs) findings correlate with high bilirubin levels. EEG and VEPs therefore may prove useful to identify and monitor neurotoxic effects of hyperbilirubinemia.

Our study is unique in paediatrics because neurological investigations of CN syndrome have been performed only in children and adolescents with type I CN. Neurophysiologic tests, in particular VEPs, may be useful for clinical management of patients with severe CN to identify and monitor neurotoxic effects of marked hyperbilirubinemia. They may contribute to choose the appropriate timing for addressing CN patients to OLT evaluation before catastrophic neurologic impairment happens. This may be particularly useful in those CN type II cases who have very severe hyperbilirubinemia poorly responsive to phenobarbital when some hesitation for liver transplantation exists on the part of doctors and patients and/or their families as well.

Future research should address better characterization of intermediate I/II phenotype CN. More precise delineation of possible relation among individual symptoms, genetic mechanisms, neurological signs and therapy response should be attempted to provide better targets for more tailored therapeutic interventions. Because of social aspects of jaundice among adolescents, more attention to this issue should also be paid.

4. 5. The last two studies who are part of this thesis regards two emerging complications of pediatric liver transplantation (OLT).

4. As for post-transplant erythrocytosis, the development of erythrocytosis in pediatric age has never been reported after OLT. We identified for the first time five cases (2 with high erythropoietin levels) who developed erythrocytosis in the long term follow-up. Causes of primary and secondary erythrocytosis, including mutations of erythropoietin-receptor, JAK2 and oxygen sensing pathway genes, were accurately excluded. In one patient erythropoietin production was much probably related to presence of renal cysts. In the remaining patients the mechanisms underlying erythrocytosis remains unknown. Since the rarity of erythrocytosis post-OLT (it has never been described), close collaboration between colleagues and medical institutions taking care of these patients will be a prerequisite for future identification of this disorder.

The detection of erythrocytosis post-OLT has several important clinical implications. From a clinical point of view its recognition and treatment are important for maintaining Hct in a safe range to reduce the clinical symptoms and/or to minimize the possible risk of hyperviscosity related thrombosis. From an investigational point of view, this possible OLT complication should be taken into consideration in follow-up laboratory programs of liver transplant children. The increasing knowledge about molecular mechanism and the possibility that congenital mutations concerning candidate genes of primary and secondary erythrocytosis will essentially contribute to identify potential etiological factors.

Future research should therefore address framework designs to produce a collaborative observational trial on erythrocytosis post- OLT in pediatric age. It is important to define prevalence, clinical presentation, biochemical markers and treatment of this disorder in childhood. Since molecular analysis of the most

common genes responsible of congenital erythrocytosis has been performed on the patient's lymphocytes, we propose to analyze their mutations also on hepatic tissue to evaluate if erythrocytosis derive from liver donor.

5. Regarding self-concept and psychopathological risk in liver transplantated children it is now becoming clear that the success of liver transplantation should not be measured only by survival rates. Its outcome will need to be judged by the amount of psychological aspects. We have confirmed on a larger series that OLT patients have an higher risk for behavioural and emotional disorders as compared to age matched controls with stable chronic liver disease. Lower performances in activity school have been demonstrated as well. As a consequence, our results highlight the fact that understanding of the interaction between OLT and patient's psychopathological risk and long-term health needs after liver transplantation is necessary. This may help in offering anticipatory guidance and targeted interventions, such as a school special support and psychological guidance. Our study might also be useful to create specific competences related to a integrated pediatric-psychosocial approach to liver transplanted children.

Future research should try to identify methods to better discover OLT patients at risk for psychopathological complications. Integrations of psychological interventions in usual therapeutic protocols of liver transplantated children are needed as well. Finally, because pediatric OLT seems to have a negative impact on some aspects of quality of life, this aspect also needs to be explored. Existing data are uncertain because of the small number of studies conducted in short-term follow-up, and their variable quality.